Comparison of detection methods of EGFR T790M mutations using plasma, serum, and tumor tissue in EGFR-TKI-resistant non-small cell lung cancer

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Background: Osimertinib, a third-generation epidermal growth factor receptor-tyrosine kinase inhibitor, exerts remarkable effects against EGFR T790M resistance mutation-positive non-small cell lung cancer. Identifying T790M mutation by re-biopsy is essential before prescribing osimertinib. Tissue biopsy is the golden standard for this purpose, but several factors limit its success rate. The liquid biopsy with blood, using circulating tumor DNA, has been an alternative method. However, the true biological meaning and equivalence of liquid biopsy and tumor biopsy are still under investigation. Especially, the usefulness of serum samples to detect T790M mutation is not yet been known.

Patients and methods: We prospectively evaluated the sensitivity, specificity, and parallelism of the detection of EGFR mutations in tissue re-biopsy and liquid biopsy (plasma and serum), simultaneously, from June 2016 to May 2017. EGFR mutations in tumor re-biopsy were evaluated by COBAS ver2 and PNA-LNA PCR clamp method, and those in liquid biopsy were evaluated with COBAS ver2.

Results: Fifteen patients were enrolled. In 10 patients whose EGFR mutation was detected in liquid biopsy, the original EGFR mutation (exon 19 del or L858R) was detected in all patients. Detection of EGFR mutation by COBAS ver2 and by PNA-LNA method was almost the same in tissue re-biopsy. The detection rate of T790M was lower than that of the original EGFR mutation in liquid biopsy compared to that in tissue re-biopsy. The detection of T790M in serum exhibited a higher specificity (67%) and positive predictive value (50%) than that in plasma (50% and 40%, respectively). The detection sensitivity was similar in plasma and serum.

Conclusion: Plasma, serum, and tissue genotyping can have complementary roles for detecting EGFR-T790M using COBAS ver2. Repeated tests with different samples and different methods may improve accuracy of T790M detection and will lead to the maximum benefit for the patient.

Keywords: EGFR, T790M mutation, liquid biopsy, COBAS ver2, PNA-LNA PCR clamp method, osimertinib

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for around 85% of all lung cancers, and majority of patients with NSCLC present with locally advanced or metastatic disease.1-3 The discovery of epidermal growth factor receptor (EGFR) mutations in NSCLC led to a new treatment paradigm. Prospective clinical trials of first- or second-generation (1st and 2nd) EGFR-tyrosine kinase inhibitors (TKIs) demonstrated remarkable response rates of approximately 70%.4-10 However, lung cancer cells inevitably
acquire resistance to these inhibitors after approximately 1 year.\textsuperscript{4,11} The major mechanism of acquired resistance is the EGFR T790M gatekeeper mutation, which accounts for approximately half of all acquired resistance to 1st- and 2nd-generation EGFR-TKIs.\textsuperscript{12–14} Recently, third-generation EGFR-TKIs such as osimertinib have been shown to exert remarkable effects against EGFR T790M mutation-positive NSCLC. Third-generation EGFR-TKIs can bind irreversibly to the EGFR kinase by targeting the cysteine-797 residue in the ATP binding site via covalent bond formation.\textsuperscript{15} Therefore, identifying T790M mutation is very important for appropriate treatment.\textsuperscript{16–18} Re-biopsy could provide further information regarding the genetic profile of tumors and may help in identifying patients eligible for treatment.\textsuperscript{19} Tissue biopsy is the golden standard method for re-biopsy, but several factors limit its success rate such as the difficulty of accessing tumor sites and the invasive nature of the sampling methods.\textsuperscript{20–25} Given these limitations, the use of circulating tumor DNA (ctDNA), commonly named “liquid biopsy”, has emerged as a new promising and less invasive tool to detect EGFR mutations in patients with NSCLC and can provide similar molecular information as those obtained by invasive tumor biopsies.\textsuperscript{26}

In June 2016, the US Food and Drug Administration approved COBAS ver2 using plasma specimens as a companion diagnostic test for the detection of exon 19 deletions or exon 21 substitution mutations in the EGFR gene, and liquid biopsy for the detection of T790M has been acknowledged as one of the re-biopsy methods in Japan since December 2016. Conversely, peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method is a laboratory-developed test to detect EGFR mutations, including T790M. Although its sensitivity is relatively high, no study has clarified which method, PNA-LNA PCR method or COBAS ver2 method, has a higher detection rate of T790M mutation in tissue and blood.

Although the usefulness of liquid biopsy for detecting EGFR mutations has been recognized, the true biological meaning and equivalence of the detection methods are still under investigation. Especially, the usefulness of serum samples to detect T790M mutation has not yet been clarified.

In this study, we prospectively evaluated the sensitivity, specificity, and parallelism of the detection of EGFR mutations in tissue re-biopsy and liquid biopsy (plasma and serum) simultaneously. EGFR mutations in tumor re-biopsy were evaluated with COBAS ver2 and PNA-LNA PCR clamp method and those in liquid biopsy were evaluated with COBAS ver2 using plasma and serum. EGFR mutation detection in liquid biopsy was carried out by BML Inc. (Tokyo, Japan).

The amount of blood needed for plasma analysis was approximately 12 mL with EDTA as an anticoagulant. Plasma was separated from the blood cells at 4°C via low-speed centrifugation (1,200×g for 15 min) within 4 hours after blood draw. The pre-analytical (sample collection and processing) protocols for serum samples are simpler than those for plasma. Approximately 9 mL of blood was harvested in a normal test tube and the serum was separated from blood cells at room temperature using the same centrifugation method. These liquid samples were stored at −80°C until DNA extraction.

Clinical characteristics

The following clinical characteristics were included in our analyses: age, sex, smoking status, the presence or absence of bevacizumab before 2nd biopsy, treatment duration with EGFR-TKIs (gefitinib, erlotinib, and afatinib), interval between diagnosis and 2nd biopsy, and the number of metastases before re-biopsy. To determine the number of

Patients and methods

Patients

From June 2016 to May 2017, we enrolled patients with advanced or recurrent EGFR-mutant NSCLC who underwent re-biopsy (2nd biopsy) based on physician’s decision in the cases of acquired resistance to 1st- or 2nd-generation EGFR-TKI treatment. All tissue samples were obtained from sites assessed as disease progression by imaging. All patients were enrolled in the Keio University Hospital. Acquired resistance was defined according to Jackman’s criteria.\textsuperscript{27} Written informed consent was obtained from all patients. We also obtained consent from patients regarding the publication of images. This study was approved by the Ethical Review Board Committee of Keio University. Research was conducted in accordance with the 1964 Declaration of Helsinki, as revised in 2013.

Tissue re-biopsy and liquid biopsy

We prospectively evaluated the sensitivity, specificity, and parallelism of the detection of EGFR mutations in tissue re-biopsy and liquid biopsy (plasma and serum) simultaneously. EGFR mutations in tumor re-biopsy were evaluated with COBAS ver2 and PNA-LNA PCR clamp method and those in liquid biopsy were evaluated with COBAS ver2 using plasma and serum. EGFR mutation detection in liquid biopsy was carried out by BML Inc. (Tokyo, Japan).

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Clinical characteristics

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metastatic sites, we counted “only one rib metastasis” as “one bone metastasis” and “one rib, one iliac, and one skull metastasis” as “three bone metastases.”

Efficacy of osimertinib

Responsiveness to osimertinib was evaluated according to the Response Evaluation Criteria in Solid Tumors (version 1.1).28

Statistical analysis

All categorical variables were analyzed by Fisher’s exact test or Student’s t-test, as appropriate. All p-values <0.05 were considered statistically significant. Statistical analyses were conducted by using JMP software (version 13; SAS Institute, Cary, NC, USA). Data were visualized by using Easy R (Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).29

Results

Patient characteristics

Fifteen patients with NSCLC harboring EGFR mutations were enrolled in this study. They underwent re-biopsy after acquiring resistance to EGFR-TKIs. Patient characteristics are shown in Table 1. The median age was 62 years, and majority of the original EGFR mutations found were exon 19 deletion (53%) and exon 21 L858R (33%).

Fourteen patients were treated with erlotinib, four patients with gefitinib, and four patients with afatinib. Seven patients were treated with double EGFR-TKIs. Eleven patients were treated with erlotinib as the initial EGFR-TKI. Nine patients were treated with bevacizumab as the initial EGFR-TKI. Nine patients were treated with erlotinib as the initial EGFR-TKI. Nine patients were treated with bevacizumab sometime before re-biopsy.

Ten patients presented positive results for EGFR mutations (exon 19 deletion, L858R, or T790M) in the liquid biopsy (plasma or serum) (Figure 1). Of these patients, only five had a positive result for T790M with the liquid biopsy. Eight patients had a positive result for T790M with tissue re-biopsy.

Differences in the methodology between the first tumor tissue biopsy and re-biopsy

There were differences in the methods used between the first biopsy (ie, at the time of first diagnosis) and re-biopsy (Table S1). CT-guided needle biopsy was used more frequently in re-biopsy than for the first biopsy.

Assay characteristics

In 10 patients whose EGFR mutation was detected in liquid biopsy, the original EGFR mutation (exon 19 deletion or L858R) was detected in all patients: both with plasma and serum. COBAS ver2 and PNA-LNA PCR clamp method exhibited 100% sensitivity, specificity, and positive predictive value for the detection of EGFR exon 19 deletion (7/7) and L858R (3/3) when compared with tumor genotyping of the re-biopsy tissue. The results of EGFR mutation detection in tissue re-biopsy using COBAS ver2 and PNA-LNA PCR clamp method were consistent, except for one case presenting a positive result with PNA-LNA PCR clamp method, but not with COBAS ver2 (Figure 2).

By contrast, the detection rate of T790M in liquid biopsy (50%) was lower than that of major EGFR mutation (exon 19 del or L858R, 100%) (Table S2).

The detection of T790M mutation in serum exhibited a higher specificity (67% [4/6]) and positive predictive value (50% [2/4]) than that in plasma (50% [3/6], 40% [2/5], respectively). The sensitivity for the detection of T790M was similar when using plasma and serum (50% [2/4]).

Clinical factors related to T790M prevalence in liquid biopsy and tissue re-biopsy

When considering the results of the liquid biopsy, the number of metastases was significantly higher in the T790M-positive
group than in the T790M-negative group in both plasma and serum (Table 2). By contrast, in tissue re-biopsy, longer duration of erlotinib treatment, but not the total duration of EGFR-TKI treatment or the number of metastases, was significantly related to T790M-positivity (Table 3).

Efficacy of osimertinib
Nine patients were treated with osimertinib. The response rate was 77.8% (7/9) and disease control rate was 100% (9/9) (Figure 2). A response was observed in patients with T790M mutation detected in both tissue and liquid biopsy, only in tissue, and only in liquid biopsy. One patient was positive for T790M in liquid biopsy (both plasma and serum) and negative in tissue re-biopsy (transbronchial lung biopsy). After 2 months of gefitinib treatment, another re-biopsy of liver metastasis was performed and was positive for T790M. Afterward, the patient was treated with osimertinib and presented a response in both the lung and liver metastases (Figure 3).

Discussion
In the Phase III LUX-Lung 3/6 trials in patients with EGFR mutation-positive lung adenocarcinoma, EGFR mutation detection rates in ctDNA were 28.6% (serum) and 60.5% (plasma) by using RT-PCR (scorpion-ARMS) method.30 Previous studies suggested that the plasma may be a more reliable source than the serum for ctDNA owing to higher background levels of non-tumor wild-type DNA found in serum. Specifically, serum yielded more cell free DNA than plasma, but the absolute quantity of mutation was higher in plasma, which may explain the differences in detection.31–33 These reports suggested that the sensitivity of the detection of EGFR mutation in the serum is lower than that in the plasma when tumor re-biopsy is used as a reference. We prospectively performed the current study to elucidate the difference in the usefulness of plasma and serum. It is known that the detection of T790M mutation after acquired resistance is always accompanied by the original EGFR mutation.34 We detected high concordance in terms of the sensitivity of EGFR mutation detection between plasma and tissue.

Figure 1 Patient flow diagram.

**Note:** L(−) denotes no mutation detected in the liquid biopsy for both original mutations and T790M.

**Abbreviations:** T, tissue; L, liquid biopsy.
Table 2 Univariate analyses of patient characteristics and T790M prevalence (plasma and serum)

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>T790M+ (plasma)</th>
<th>T790M− (plasma)</th>
<th>p-value</th>
<th>T790M+ (serum)</th>
<th>T790M− (serum)</th>
<th>p-value</th>
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<td>Duration of erlotinib treatment, m</td>
<td>7.6±3.9</td>
<td>11±3.9</td>
<td>0.56</td>
<td>8.5±4.5</td>
<td>9.8±3.7</td>
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<td>Duration of all EGFR-TKis treatment, m</td>
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<td>24.1±5.6</td>
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<td>16.3±9.0</td>
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<td>Interval between diagnosis and re-biopsy, m</td>
<td>31.2±6.2</td>
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<td>Number of metastases, n</td>
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<td>2.0±0.8</td>
<td>0.006</td>
<td>6.5±1.0</td>
<td>2.5±0.8</td>
<td>0.013</td>
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</table>

Note: Data are shown as mean ± SD.
Abbreviations: EGFR-TKis, epidermal growth factor receptor-tyrosine kinase inhibitors; m, months.

Table 3 Univariate analyses of patient characteristics and T790M prevalence in tissue biopsy

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>T790M+ (tissue)</th>
<th>T790M− (tissue)</th>
<th>p-value</th>
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<td>Age, n</td>
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<td>4</td>
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<tr>
<td>Bevacizumab as prior therapy, n</td>
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<td>6</td>
<td>3</td>
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<tr>
<td>Duration of erlotinib treatment, m</td>
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<td>6.3±2.4</td>
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<td>Duration of all EGFR-TKis treatment, m</td>
<td>19.9±5.6</td>
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<td>Interval between diagnosis and re-biopsy, m</td>
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<tr>
<td>Number of metastases, n</td>
<td>19.9±5.3</td>
<td>18.9±5.6</td>
<td>0.9</td>
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</table>

Note: Data are shown as mean ± SD.
Abbreviations: EGFR-TKis, epidermal growth factor receptor-tyrosine kinase inhibitors; m, months.
because tumor tissue re-biopsy was negative for T790M, it might be useful to rely on the result of both serum and plasma samples. We suggest that the combined results of mutation detection using serum and plasma could provide insights into the efficacy of osimertinib treatment.

The major limitation of our study was the small sample size. Further enrollment of cases is necessary. However, we believe that the heterogeneous patient population in our study may be very important and represent population variability, resembling other studies’ populations. First, the number of metastases was significantly higher in the T790M-positive group than in the T790M negative group (Table 2). Thress et al reported that T790M mutation was more readily detected in the plasma of patients with extra-thoracic metastatic disease than in that from patients with disease confined to the thoracic cavity (as determined by droplet digital PCR assay). This suggests that tumor bulk and metastatic status may impact on the presence EGFR mutation in the plasma and should be further validated in clinical settings. We also found that the duration of erlotinib treatment was significantly longer in the T790M-positive group than in the T790M-negative group. Several reports analyzed the relationship between T790M induction and exposure to EGFR-TKIs in preclinical and clinical studies. We do not have an explanation as to why only the duration of erlotinib treatment, but not the total duration of the TKI treatment, is related to T790M positivity. One possibility may be that the total duration includes “beyond PD” treatment, ie, EGFR-TKI treatment after progression, and may not relate to the induction of T790M. Further studies are needed to confirm this possibility.

Finally, we found a case with discordant results of T790M detection in tissue re-biopsy using COBAS ver2 and PNA-LNA PCR clamp method. Previous studies reported that the detection rate of EGFR mutation is higher with the PNA-LNA PCR clamp method than with COBAS ver2. Although the companion diagnostic is COBAS ver2, one might consider using a different method like PNA-LNA PCR clamp method to confirm the T790M negative results by COBAS ver2, to detect T790M, in order to not directly rule out the possibility of osimertinib in T790M-negative cases by COBAS ver2.

**Conclusion**

Our data suggest that plasma, serum, and tissue genotyping can have complementary roles for T790M testing by using COBAS ver2 and the PNA-LNA PCR clamp method. Especially, the result of both serum and plasma samples for T790M detection could be more useful to predict the efficacy
of osimertinib than only plasma samples when tissue genotyping is negative for T790M or tissue genotyping is unknown.

Acknowledgments
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Author contributions
KK participated in the acquisition of data, analysis and interpretation of data, and drafting the manuscript and revising it. KN participated in the conception and design, acquisition of data, analysis and interpretation of data, and drafting the manuscript and revising it. TM, KM, HH, HY, IK, KS, and TB participated in the interpretation of data and drafting the manuscript and revising it. All the authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

References

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Supplementary materials

Table S1 Comparison between the first biopsy and re-biopsy

<table>
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<th>Site of biopsy</th>
<th>First biopsy</th>
<th>Re-biopsy</th>
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Abbreviations: CNS, central nervous system; CT-NB, computed tomography-guided needle biopsy.

Table S2 Plasma and serum assay sensitivity, specificity, and positive predictive value

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<th>Mutation</th>
<th>Sensitivity, %</th>
<th>True positive, n</th>
<th>False negative, n</th>
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