Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor

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A secondary epidermal growth factor receptor (EGFR) mutation, the substitution of threonine 790 with methionine (T790M), leads to acquired resistance to reversible EGFR-tyrosine kinase inhibitors (EGFR-TKIs). A non-invasive method for detecting T790M mutation would be desirable to direct patient treatment strategy. A total of 75 NSCLC patients with progressive disease (PD) after undergoing EGFR-TKI treatment (gefitinib or erlotinib) at the Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research between 2006 and 2011 were included in this study. Progressive disease was defined as the appearance of a new lesion or a 20% increase in the size of a primary tumor. The period between the detection of PD and the collection of the plasma sample was defined as the appearance of PD. The plasma samples obtained after discontinuation of EGFR-TKI were identified using direct sequencing. A secondary epidermal growth factor receptor (EGFR) mutation, the substitution of threonine 790 with methionine (T790M), leads to acquired resistance to reversible EGFR-TKIs. The most common known mechanism to EGFR-TKIs is the substitution of threonine 790 with methionine (T790M), leading to acquired resistance to reversible EGFR-TKIs. The T790M mutation after EGFR-TKI treatment; and (ii) the association between the T790M mutation status and the clinical outcome. The detection of the T790M mutation in plasma samples could enable the clinical response to EGFR-TKIs to be monitored.

Materials and Methods

Patients. A total of 75 NSCLC patients with progressive disease (PD) after undergoing EGFR-TKI treatment (gefitinib or erlotinib) at the Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research between 2006 and 2011 were included in this study. Progressive disease was defined as the appearance of a new lesion or a 20% increase in the size of a primary tumor. The period between the detection of PD and the collection of the plasma sample was defined as the appearance of PD. The plasma samples obtained after discontinuation of EGFR-TKI were identified using direct sequencing. Table 1 shows the clinical characteristics of the patients. The plasma samples obtained after discontinuation of EGFR-TKI were used to examine the T790M mutation status. All the patients provided informed written consent, and the study was approved by the Institutional Review Board at the Cancer Institute Hospital and the Kinki University Faculty of Medicine.

Sample processing. Plasma DNA was purified using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). The extracted DNA was stored at −80°C until analysis.

Assay design. The assay was designed using MassARRAY Assay Design 4.0 software (Sequenom, San Diego, CA, USA) with a slight modification to enable use with the SABER method; the assay was intentionally designed so that it would only include terminators for the mutated nucleotide, and not the terminator for the wild-type nucleotide. A schematic diagram of the assay is shown in Figure 1. An amplification control assay was incorporated into each reaction using a conserved sequence in the amplified EGFR transcript so that...
amplification would always occur in the presence of input template DNA. The PCR and extension primer sequences are listed in Table 2.

**SABER method.** The SABER method, where the primer extension was restricted to the mutant-specific allele, was performed using Sequenom iPlex Pro biochemistry with resultant products detected with the MassARRAY platform. The PCR reactions were performed in 5-μL volumes containing 1.5 μL of eluted serum DNA, 200 nM of each primer, 50 μM of dNTPs (Sequenom), 0.2 U of PCR Taq DNA polymerase (Sequenom), 2.0 mM of MgCl₂, and 1× PCR buffer (Sequenom). The thermal cycling for the PCR was performed as follows: 2 min at 94°C, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The program was terminated after a final incubation at 72°C for 5 min. After the completion of the PCR, 2 μL (0.5 U) of shrimp alkaline phosphatase (Sequenom) was added to each reaction and the resulting mixture was incubated for 40 min at 37°C before enzyme inactivation by incubating the sample for 5 min at 85°C. The single-base primer extension reaction (SABER) was then performed in a final volume of 9 μL, containing 1 μmol of each extension primer, a mixture of three iPLEX mass-modified terminators (Sequenom), and 2.56 U of PCR, polymerase chain reaction.

**Fig. 1.** Schematic diagram of the T790M-SABER method, based on MassARRAY assays. The DNA samples were first amplified using polymerase chain reaction (PCR). The PCR products were then subjected to a single base extension reaction. The T790M-SABER reaction only included terminators for the mutated nucleotide, and not the terminators for the wild-type nucleotide. An internal amplification control was designed to prevent false-negative results caused by PCR inhibitors. The primer extension products were analyzed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). UEP, unextended primer.

| Table 1. Clinical characteristics of patients with progressive disease (PD) after epidermal growth factor-tyrosine kinase inhibitor (EGFR-TKI) treatment (n = 75) |
|-----------------|-----------------|-----------------|-----------------|
| EGFR-TKI-treated Patients | All patients (n = 75) No. (%) | Post-treatment T790M mutation positive patients (n = 21) | Post-treatment T790M mutation negative patients (n = 54) |
| Age, years (mean, 61.6 ± 8.5 years) ≤65 | 47 (62.7) | 11 | 36 |
| >65 | 28 (37.3) | 10 | 18 |
| Sex Male | 21 (28.0) | 7 | 14 |
| Female | 54 (72.0) | 14 | 40 |
| Smoking No | 44 (58.7) | 12 | 32 |
| Yes | 31 (41.3) | 9 | 22 |
| Histology Ad | 71 (94.7) | 19 | 52 |
| Large/Sq | 4 (5.3) | 2 | 2 |
| EGFR mutation (pre-treatment, tumor) Activating mutation | 60 (80.0) | 18 | 42 |
| Wild type/unknown | 15 (20.0) | 3 | 12 |
| Response to EGFR-TKI PR | 60 (80.0) | 20 | 40 |
| SD/PD/NE | 15 (20.0) | 1 | 14 |

†Fisher’s exact test. Ad, adenocarcinoma; Large, large cell carcinoma; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma.

| Table 2. Primers used in the T790M SABER (single allele base extension reaction) method |
|-----------------|-----------------|
| Sequences |
| PCR Forward | 5′- ACGTGGAGATGCTGCCTCACCTCATGCCTCGG -3′ |
| Reverse | 5′- ACGTGGAGATGCTGCCTCACCTCATGCCTCGG -3′ |
| Extension T790M | 5′- CACCAGACGGCTCATCA -3′ |
| Internal control | 5′- GTCCAGGGAGCCAGCCGAAG -3′ |

PCR, polymerase chain reaction.
ThermoSequenase (Sequenom). The thermal cycling program for the reaction included an initial denaturation for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. Forty additional annealing and extension cycles (5 s at 94°C, 5 s at 52°C, and 5 s at 80°C) were then performed. The final extension was performed at 72°C for 3 min, and the samples were then cooled to 4°C. The reaction products were desalted by dilution with 41 μL of distilled water, the addition of 15 mg of ion-exchange resin (Sequenom), and subsequent separation of the resin by centrifugation. The products were spotted on a SpectroChip II (Sequenom), processed, and analyzed using a Compact Mass Spectrometer and MassARRAY Workstation (version 3.3) software (Sequenom). The data analysis was performed using MassARRAY Typer software, version 4.0 (Sequenom).

Sequencing analysis. The PCR products were subcloned into a pTA2 vector (Toyobo, Osaka, Japan) and sequenced using an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) and M13 universal primers (Applied Biosystems).

Scorpion ARMS analysis. Scorpion ARMS analysis used the DxS EGFR Mutation Test Kit for research use only (Qiagen) and was carried out according to the manufacturer’s instructions.

Statistical analyses. The Fisher exact test was used to assess the relationship between the T790M mutation status and different clinical characteristics, including patient sex and the primary mutation status. The objective tumor response (partial response [PR], stable disease [SD], or progressive disease [PD]) was evaluated according to the Response Evaluation Criteria in Solid Tumors guidelines. Progression-free survival (PFS) was defined as the period from the start of treatment until the date when disease progression was observed. The survival curves were derived using the Kaplan–Meier method and were compared using the log-rank test. All the statistical analyses were performed using JMP software (version 10; SAS Institute, Cary, NC, USA). A \( P \)-value < 0.05 was considered statistically significant.

Results

Assay sensitivity. The assay sensitivity was determined using T790M DNA oligonucleotide \((5.0 \times 10^{-10}, \ 2.5 \times 10^{-10}, \ 1.3 \times 10^{-10}, \ 6.3 \times 10^{-11}, \ 3.1 \times 10^{-11}, \ 1.6 \times 10^{-11})\), and

Fig. 2. Sensitivity of T790M detection. The percentages indicate the calculated proportion of T790M DNA in a mixture with wild-type DNA. An arrow at 5442 Da indicates the detection of the T790M mutation. An asterisk indicates a non-specific background peak. Three independent experiments were performed in duplicate, with identical results. DW, distilled water; UEP, unextended primer.
Figure 2 shows the MALDI-TOF MS spectra. Concentrations as low as $3.1 \times 10^{-17}$ mol of T790M DNA mixed with $1 \times 10^{-14}$ mol of wild-type DNA could be detected, indicating a detection sensitivity of approximately 0.3%, which is in agreement with previous studies using SABER.\(^{(6)}\)

Detection of T790M in plasma DNA. We examined the T790M mutation status in plasma samples obtained after dis-
continuation of EGFR-TKI. Using the SABER method, the internal control was successfully amplified and detected in all the samples (data not shown). The T790M mutation was detected in 21 of the 75 samples (28%). The key results of the SABER method are shown in Figure 3. The clinical characteristics of the T790M-positive patients are shown in Table 3.

The presence of the T790M mutation was confirmed by subcloning into sequencing vectors and sequencing. When up to 105 colonies were selected and sequenced (theoretical median limit of detection of 0.95%), the T790M mutation was confirmed in 14 of the 21 (66.6%) PCR products. The T790M mutation in plasma was also identified by the Scorpion ARMS (Table S1).

Fig. 4. Progression-free survival according to T790M mutation status as measured in plasma DNA. The Kaplan–Meier progression-free survival curves are shown for all the T790M-positive and T790M-negative patients (n = 75) in (a), for patients aged 65 years or younger (n = 47) in (b), for patients with a partial response to epidermal growth factor-tyrosine kinase inhibitors (EGFR-TKIs) treatment (n = 60) in (c), and for patients aged 65 years or younger who had a partial response to EGFR-TKI treatment (n = 40) in (d).
T790M was detected in 6/75 cases (8%). Of these cases, 5/6 were also positive by the SABER method. Compared to the positive rate (28%, 21/75) when using the SABER method, the rate detected by the Scorpion ARMS was relatively low.

Detection of T790M in pretreatment tumor specimens. We analyzed the T790M mutation status in the tumor samples using the SABER method. All of the tumor samples were obtained before the EGFR-TKI treatment. No tumor sample was obtained by the re-biopsy after EGFR-TKI treatment. T790M was detected in two tumor samples (Table S1). One case (no. 185) was double positive (tissue +, plasma +). Another case (no. 167) was T790M positive in tumor samples only. The positive rate (7%, 2/28) was relatively low as compared with that of plasma samples (46%, 13/28). These data suggest that the T790M mutation detected in the plasma is acquired by EGFR-TKI treatment and that detection of T790M in the plasma is feasible to detect EGFR-TKI refractory cases.

Correlation between plasma T790M mutation status and clinical outcome. There was no significant difference in clinical characteristics between patients with or without T790M mutation (Table 1). T790M positive detection tends to be observed more frequently in PR patients compared with non-PR patients although the correlation is not highly significant (P = 0.053, Table 1).

In our cohort of 75 patients, the median PFS of the patients with the T790M mutation (n = 21) was not statistically different from that of the patients without the mutation (n = 54) (P = 0.9443), being 289 days and 210 days, respectively (Fig. 4a). When patients aged 65 years or younger were subdivided into two groups according to their plasma T790M status, the median PFS of the T790M-positive patients (n = 11) tended to be shorter than that of the T790M-negative patients (n = 36, P = 0.1486; Fig. 4b). We also compared the PFS of patients according to their response to the EGFR-TKIs and found no statistical differences between the responders (PR) and the non-responders (P = 0.4303; Fig. 4c). When patients aged 65 years or younger who had a PR were grouped according to their plasma T790M mutation status, the PFS of the T790M-positive patients (n = 11) was significantly shorter than that of the T790M-negative patients (n = 29, P = 0.0325; Fig. 4d) being 289 days and 391 days, respectively.

Discussion

Our findings show that the T790M mutation can be detected in plasma samples obtained after discontinuation of EGFR-TKI and that the SABER method is a feasible means of determining the plasma T790M mutation status. We detected the T790M mutation in 21 out of 75 plasma samples that were obtained from patients after discontinuation of EGFR-TKI (28%). This frequency seems to be lower than the positive rate (~50%) in tumor tissue samples reportedly. However, the positive rate when using SABER is relatively higher than when using Scorpion-Arms (Table S1) for the same samples and our previous report. We can speculate that the sensitivity of the SABER assay for circulating samples (plasma or serum) is much improved. In this study, plasma samples were collected following therapy with the EGFR-TKI; therefore, the time between the detection of PD and that of sample collection was varied. To conclude whether this sensitivity is enough or not, it will be necessary to conduct a prospective comparison study using the paired samples of plasma and re-biopsy samples as the next step. Previously, we attempted to determine the EGFR mutation status in serum samples using the highly sensitive Scorpion-Arms method. In that cohort, a high false-negative rate was observed, and more sensitive methods of detecting EGFR mutations in serum samples are desirable. Another group reported the results of a serum EGFR mutation analysis using the MBP-QP method which yielded a detection sensitivity equivalent to that of the SABER method used in the present report. Automated and high throughput analysis is an advantage of SABER method.

Direct sequencing of the subcloned PCR products confirmed the presence of the T790M mutation, suggesting that this SABER method is highly specific. However, the clonal analysis did not detect the T790M mutation in seven of the 21 (33.3%) samples that were found to be T790M-positive using SABER. In this cohort, we selected and sequenced up to 105 colonies (theoretical median limit of detection of 0.95%). Therefore, the number of tested colonies might not have been large enough to detect the low frequency of mutant clones with normal sampling error distribution.

In our study, the median PFS of the T790M-positive patients was significantly shorter than that of the T790M-negative patients with a clinical PR among younger (≤55 years) patients. It is likely that the prognosis of patients with an activating mutation who acquire a resistance mutation is less favorable. However, Oxnard et al. showed that EGFR-TKI resistant patients with T790M identified in re-biopsy specimens had a relatively favorable prognosis compared with patients without the T790M mutation. Their data seems to be inconsistent with our result. Different materials and detection methods may be the cause of this discrepancy between two studies.

The early detection of T790M mutation may be beneficial to such patients.

The intrinsic existence of T790M clones in pretreatment tumors has been previously suggested. Indeed, the T790M mutation has been detected in a few pretreatment tumor samples. The T790M-SABER method is highly sensitive and is capable of detecting this mutation in plasma samples; consequently, the detection of the T790M mutation before or during EGFR-TKI treatment may be possible. Consequently, the T790M-SABER method is a promising tool for the detection of T790M mutation in a diagnostic setting.

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Disclosure Statement

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References


3 Kasahara K, Arao T, Sakai K et al. Impact of serum hepatocyte growth factor on treatment response to epidermal growth factor receptor tyrosine kinase

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. A comparison of T790M detection by SABER and Scorpion-ARMS methods.