Next-generation sequencing of tyrosine kinase inhibitor-resistant non-small-cell lung cancers in patients harboring epidermal growth factor-activating mutations

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Abstract

Background: The aim of this study was to detect the epidermal growth factor receptor (EGFR)-activating mutations and other oncogene alterations in patients with non-small-cell lung cancers (NSCLC) who experienced a treatment failure in response to EGFR-tyrosine kinase inhibitors (TKIs) with a next generation sequencer.

Methods: Fifteen patients with advanced NSCLC previously treated with EGFR-TKIs were examined between August 2005 and October 2014. For each case, new biopsies were performed, followed by DNA sequencing on an Ion Torrent Personal Genome Machine (PGM) system using the Ion AmpliSeq Cancer Hotspot Panel version 2.

Results: All 15 patients were diagnosed with NSCLC harboring EGFR-activating mutations (seven cases of exon 19 deletion, seven cases of L858R in exon 21, and one case of L861Q in exon 21). Of the 15 cases, acquired T790M resistance mutations were detected in 9 (60.0 %) patients. In addition, other mutations were identified outside of EGFR, including 13 cases (86.7 %) exhibiting TP53 P72R mutations, 5 cases (33.3 %) of KDR Q472H, and 2 cases (13.3 %) of KIT M541L.

Conclusions: Here, we showed that next-generation sequencing (NGS) is able to detect EGFR T790M mutations in cases not readily diagnosed by other conventional methods. Significant differences in the degree of EGFR T790M and other EGFR-activating mutations may be indicative of the heterogeneity of disease phenotype evident within these patients. The co-existence of known oncogenic mutations within each of these patients may play a role in acquired EGFR-TKIs resistance, suggesting the need for alternative treatment strategies, with PCR-based NGS playing an important role in disease diagnosis.

Keywords: Acquired resistance, Epidermal growth factor, Next-generation sequencing, Tyrosine kinase inhibitor
Background
Recent advances in biomedical research have provided a greater understanding of the molecular basis of disease, with significant implications for therapeutic intervention. Somatic mutations, such as epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) gene rearrangements, play a significant role in the pathogenesis of non-small-cell lung cancer (NSCLC), with treatment decisions often based upon the outcome of these genetic tests [1–5].

Both EGFR and ALK function as a receptor tyrosine kinase, which are readily inhibited by a series of tyrosine kinase inhibitors (TKI), including gefitinib [6], erlotinib [7], and crizotinib [2]. Despite the initial treatment efficacy of these TKIs for the treatment of NSCLC, acquired resistance was found to develop in almost all cases. The well-known mechanism of acquired EGFR-TKIs resistance include second site mutations within the EGFR kinase domain [8, 9], up-regulation of alternative signaling pathways, such as MET [10], histologic transformation, epithelial to mesenchymal transition, and small cell transformation [11]. Although many resistance mechanisms have been clarified, the EGFR kinase domain mutation T790M in exon 20 accounts for nearly half of all acquired resistance, making testing for this mutation a key factor in determining follow-up treatment strategies in the era of second- and third-generation EGFR-TKIs [12, 13].

The recent development of next-generation sequencing (NGS) as a diagnostic tool in the clinical setting has enabled us to determine rapid, targeted sequencing of tumors for causative mutations. When combined with various selective capture approaches, NGS has allowed for the efficient simultaneous genetic analysis of a large number of candidate genes. Here, we applied a polymerase chain reaction (PCR) based NGS in determining oncogene alternations in the state of disease progression.

PCR based next-generation sequencing is an outstanding tool to provide a comprehensive genomic diagnosis in patients with recurrent NSCLC [14]. The primary aim of this study was to evaluate EGFR T790M secondary mutations, along with other oncogenic alterations, in NSCLC patients previously diagnosed with EGFR activating mutations who experienced disease recurrence after treatment with first-generation EGFR-TKIs.

Methods
Patients and treatment regimens
Fifteen patients with NSCLC previously treated with EGFR-TKIs were examined between August 2005 and October 2014 at the Institute of Biomedical Research and Innovation in Kobe City, Japan. Patients were treated with either of erlotinib or gefitinib daily, at initial daily doses of 150 (erlotinib) and 250 (gefitinib) mg/day. Standard Response Evaluation Criteria in Solid Tumors (RECIST 1.0) was used to evaluate treatment response. Toxicities were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. We obtained written informed consents from all the participants. This study was approved by the Research Ethics Committee of the Institute of Biomedical Research and Innovation.

EGFR mutational analysis
A quantity of cancer cells sufficient for a pathologic diagnosis (i.e., several hundred cells) were obtained from formalin-fixed paraffin-embedded (FFPE) biopsy specimens by manual micro-dissection. Similar biopsy specimens were used to analyze EGFR somatic mutations in exons 18–21 [15, 16].

MET gene amplification
For each patient, DNA was extracted, and the concentration measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). MET copy number gains (CNG) analysis was performed using the One-Step Real Time PCR System (Thermo Fisher Scientific, Foster City, CA) under the following conditions: one cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The qPCR reaction mixture contained 10 μL of 2X TaqMan genotyping master mix, 1 μL of the TaqMan copy number target assay, 1 μL of the TaqMan copy number reference assay (RNase P, which is known to exist only in two copies in a diploid genome), 4 μL of nuclease-free water, and 4 μL of DNA (diluted to a concentration of 5 ng/μL). Each sample was run in a minimum of four replicates. Amplification results were then analyzed using the CopyCaller Software (Thermo Fisher Scientific) for post-qPCR data analysis. To accurately detect MET CNG, we analyzed the previous reported region of MET [17], a region spanning the intron 20–exon 21 boundary (TaqMan copy number assay Hs02849644_cn).

Ion torrent PGM library preparation and sequencing
An Ion Torrent adapter-ligated library was generated using an Ion AmpliSeq Library Kit 2.0 according to the manufacturer’s protocol (Thermo Fisher Scientific, Rev. 5; MAN0006735). Briefly, 50 ng of pooled amplicons and the Ion AmpliSeq Cancer Hotspot Panel version 2 (Thermo Fisher Scientific) were end-repaired, and Ion Torrent adapters P1 and A were ligated using DNA ligase. Following AMPure bead (Beckman Coulter, Brea, CA, USA) purification, the concentration and size of the library were determined using the Life Technologies StepOne system (Thermo Fisher Scientific) and Ion Library TaqMan quantitation assay kit (Thermo Fisher Scientific). Sample emulsion PCR, emulsion breaking, and enrichment were
performed using the Ion PGM IC 200 Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Briefly, an input concentration of one DNA template copy/Ion Sphere Particle (ISP) was added to the emulsion PCR master mix, and the emulsion was generated using the Ion Chef (Thermo Fisher Scientific). Next, ISPs were recovered and template-positive ISPs enriched using Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher Scientific). Sequencing was undertaken using 314 BC chips on the Ion Torrent PGM for 65 cycles using barcoded samples. The totally turnaround time from library preparation to the end of sequencing is about 2 days.

**Variant calling**

After sequencing, data were processed using the Ion Torrent platform-specific pipeline software Torrent Suite to generate sequence reads, trim adapter sequences, and remove poor signal-profile reads. Initial variant calling was generated using Torrent Suite Software v4.0 using the variant caller plug-in. To eliminate erroneous base calling, three filtering steps were used. The first filter was set at an average total coverage depth of >100, variant coverage of >20, and P values <0.01. The second filter was employed by visually examining mutations using the Integrative Genomics Viewer (http://www.broadinstitute.org/igv) or CLC Genomics Workbench version 7.04 (Qiagen) software. Finally, possible strand-specific errors, such as mutation only detected in only the plus or minus strand were removed.

**Results**

A summary of patient characteristics can be found in Table 1. All patients were Japanese, consisting of 10 females (76.7 %) and 5 males (33.3 %). Nine patients (60.0 %) were never smokers, and the remaining six patients (40.0 %) were former smokers. All patients had stage IV adenocarcinoma, as defined based upon TNM classification criteria (7th edition) [18]. Eight patients received erlotinib, and four patients were treated with gefitinib. The remaining patient was treated first with gefitinib, then switched to erlotinib. The median duration of EGFR-TKI therapy was 510 days (range: 122–1912 days; Table 1). 

EGFR sequence variations are listed in Table 2. All patients were diagnosed with adenocarcinomas harboring EGFR activating mutations (seven cases of exon 19 deletion, seven cases of L858R in exon 21, and one case of L861Q in exon 21). Of the 15 cases, acquired EGFR T790M resistance mutations in exon 20 were detected in 9 (60.0 %) patients. Of particular interest were cases 7, 8, and 10, in which T790M mutations were not detected by high-sensitivity conventional PCR-based methods, such as peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp [16], or Cycleave real-time PCR [15]. In addition to T790M mutations, a large number of activating mutations were identified outside of EGFR. MET amplification, another common mutation associated with EGFR-TKI resistance, was not seen (Fig. 1), which is also confirmed by copy number analysis of NGS sequencing data (data not shown). Further screening of an additional 50 known oncogenes revealed a quite number of mutations in at least 32 genes (Table 3), including 13 cases (86.7 %) of TP53 P72R mutations, 5 cases (33.3 %) of KDR Q472H, and 2 cases (13.3 %) of KIT M541L. A full list of genes analyzed in this study is shown in Table 4.

**Discussion**

In this study we analyzed biopsy specimens of patients who underwent second biopsy after treatment failure with the first generation EGFR-TKIs. There was a significant difference between the frequency of EGFR T790M and other EGFR-activating mutations, with significant variability among cases (4.8–41.3 %). The existence of EGFR and other mutations within the same tumor sample identified by NGS highlights the importance of this type of analysis in guiding appropriate cancer therapy.

High-throughput sequencing was able to detect T790M mutation in a number of cases with the same accuracy of conventional highly sensitive conventional PCR methods, such as PNA-LNA PCR clamp [16] and Cycleave real-time PCR [15]. While high sensitivity and specificity of these methods is well established [19–27], the use of NGS provides important advantages with clarifying activating mutation rate in tumor sample as well as greater detection of rare mutations outside of

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**Table 1 Patient characteristics**

| Patient characteristics (%) |  |
|-----------------------------|---|
| Age (years)                 |   |
| Range                       | 54–79 |
| Gender                      |   |
| Male                        | 5 (33.3) |
| Female                      | 10 (76.7) |
| Smoking status              |   |
| Non-smoker                  | 9 (60.0) |
| Former Smoker               | 6 (40.0) |
| Stage                       |   |
| IV                          | 14 (93.4) |
| rIV<sup>a</sup>             | 1 (6.6) |
| 1<sup>st</sup> line         | 5 (33.3) |
| 2<sup>nd</sup> line         | 7 (46.7) |
| 3<sup>rd</sup> line         | 2 (13.4) |
| Subsequent therapy          | 1 (6.6) |

<sup>a</sup>rIV recurrent stage IV
target areas [28–31]. In addition, to emphasize the power of NGS in clinical practice, we should also try to develop its applications and usages such as challenging specimens or testing processes, such as peripheral blood in the future. NGS is also able to overcome issue of germ-line DNA contamination, similar to that of new PCR methods, such as digital PCR [32]. This tolerance of germ-line DNA contamination allows for more streamlined sample preparation techniques, without need for time-consuming procedures such as macro- or micro-dissection. In this study, all samples were extracted from FFPE biopsy specimens, highlighting both versatility and potential use of NGS in clinical settings. Furthermore NGS is able to quantify gene mutations within a tumor sample. Due to the unpredictability of PCR amplification and germ line DNA contamination, observed mutations does not always reflect the penetrance of a mutation within a sample. While most highly sensitive detection methods provide only categorical results such as positive and negative, our analysis was able to identify the degree of $EGFR\ T790M$ and other $EGFR$-activating mutations within a sample that could not be explained by germ-line DNA contamination and/or PCR efficacy. These results are consistent with previous reports detailing $T790M$ allelic frequency in terms of both intra-tumor heterogeneity in localized lung adenocarcinomas [33] and allelic imbalances [34]. Our analysis was able to identify the degree of $EGFR\ T790M$ and other $EGFR$-activating mutations within a sample that could not be explained by germ-line DNA contamination and/or PCR efficacy. Future treatment with next-generation EGFR-TKIs targeting $T790M$ is likely to be informed by such analyses, as

| Case | Histology | EGFR Sequence Variants | Frequency (%) | Allele Call | Exon 20 T790M | Conversion to SCLC | Prior TKIs | Duration (days) |
|------|-----------|------------------------|--------------|------------|--------------|-----------------|------------|----------------|
| 1    | Adenocarcinoma | Exon 19 E746_T750 del | 44.3         | Heterozygous | Yes          | No              | Erlotinib  | 681            |
| 2    | Adenocarcinoma | Exon 19 E746_T751 del > A | 59.4 | Heterozygous | No           | -               | No Gefitinib | 537            |
| 3    | Adenocarcinoma | Exon 21 L858R Exon 18 T725R | 46.1 | Heterozygous | No           | -               | No Gefitinib | 195            |
| 4    | Adenocarcinoma | Exon 21 L858R Exon 20 S768I | 23.3 | Heterozygous | No           | -               | No Erlotinib | 217            |
| 5    | Adenocarcinoma | Exon21 L858R Exon 18 E709G | 56.9 | Heterozygous | No           | -               | No Gefitinib | 1105           |
| 6    | Adenocarcinoma | Exon 19 E746_T750 del | 97.2         | Homozygous | Yes          | 21.8            | No         | 693            |
| 7    | Adenocarcinoma | Exon 21 L858R | 13.8         | Heterozygous | Yes          | 5.2             | No         | 537            |
| 8    | Squamous cell carcinoma | Exon 19 E746_T750 del | 86.9         | Heterozygous | Yes          | 7.3             | No         | 315            |
| 9    | Adenocarcinoma | Exon 19 E746_T750 del | 65.3         | Heterozygous | Yes          | 41.3            | No         | 1555           |
| 10   | Adenocarcinoma | Exon21 L858R Exon 19 E746_T750 del | 11.2 | Heterozygous | Yes          | 4.8             | No Gefitinib | 1912           |
| 11   | Adenocarcinoma | Exon 19 E746_T750 del | 46.4         | Heterozygous | Yes          | 11.0            | No         | 256            |
| 12   | Adenocarcinoma | Exon21 L858R Exon 19 E746_T750 del | 22.2 | Heterozygous | No           | -               | No Erlotinib | 924            |
| 13   | Adenocarcinoma | Exon 21 L611Q Exon 20 P772S Exon19 L747S Exon2 A289V | 59.9 | Heterozygous | No           | -               | No Gefitinib Erlotinib | 1304122 |
| 14   | Adenocarcinoma | Exon 19 E746_T750 del | 80.82 | Heterozygous | Yes          | 14.8            | No         | 392            |
| 15   | Adenocarcinoma | Exon21 L858R Exon 19 E746_T750 del | 76.7 | Heterozygous | Yes          | 10.3            | No         | 339            |
patients should be treated based upon their EGFR acquired mutation [35].

In addition to EGFR mutations, we also evaluated another 50 oncogenes thought to have an important role in cancer pathogenesis (Table 4). A large number of mutations were identified in this analysis. However, how much extent these genes affect tumorigenicity, tumor progression, and resistance to EGFR-TKIs is difficult to assess, as some mutations may represent only passive alterations (passenger mutations). Although many of these mutations were identified in a single patient, a series of mutations including TP53 P72R, KDR Q472R, and KIT M541L were detected in more than two cases, suggesting a role in disease progression. TP53 P72R was the most common mutation, detected in 13 of 15 cases (86.7 %). In human populations, TP53 codon 72 is encoded by the nucleotide sequence CCC, which encodes proline, or CGC, which encodes arginine. While proline is the most common amino acid found at this residue, comparative sequence analyses have detected a high degree (>50 %) of TP53-R72 variants among certain populations [36]. The current understanding of TP53 biology is that TP53-R72 is more effective at inducing apoptosis and protecting stressed cells from neoplastic development than the more common TP53-P72 [37]. However, it is not yet understood how these functional differences might translate between in vitro and in vivo settings [38, 39], making it difficult to assess the role of this sequence variant of EGFR-TKI resistance.

KDR (kinase insert domain receptor, also known as VEGFR2) is an important factor in tumor development and progression due to its pro-angiogenic effects [40]. KDR Q472H mutations were detected in 5 of 15 cases (33.3 %), making it the second most common gene variant observed outside of EGFR. In human populations, codon 472 of KDR is encoded by the nucleotide sequence CAA, which encodes glutamine, or CAT, which encodes histidine. The Q472H variant is thought to affect protein function due to increased phosphorylation after vascular endothelial growth factor (VEGF)-A stimulation, along with increased binding efficiency for VEGF-A165 [41]. The effect of Q472H on microvessel density is thought to occur as a result of increased phosphorylation of VEGFR2 [42]. Here, increased microvessel density may have contributed to EGFR-TKI resistance, suggesting that VEGFR2 inhibition may inhibition may become an important therapeutic option in patients with documented EGFR-TKI resistance.

V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (KIT) M541L substitutions were detected in 2 of 15 cases (13.3 %). c-KIT is one of the primary targets of imatinib, and mutations in KIT are predictive of the efficacy of the drug in gastrointestinal stromal tumors (GIST) [43]. Several case reports have suggested
Table 3 Coexisting somatic mutations resulting in amino-acid changes identified using the Ion AmpliSeq Hotspot Panel version 2

| Case   | Gene          | Mutation | Frequency (%) | Frequency (%) | Frequency (%) | Frequency (%) | Frequency (%) |
|--------|---------------|----------|---------------|---------------|---------------|---------------|---------------|
| Case 1 | KIT           | M541L (COSM 28026) | 70.9          | TPS3 P72R     | 53.2     | —             | —             |
| Case 2 | PTEN          | L57W (COSM 5253)  | 21.2          | —             | —             | —             | —             |
| Case 3 | TPS3          | P72R     | 57.0          | CNTN1 D32N (COSM 5672) | 34.5 | TPS3 V73 del | 29.1          | CDH1 Q346* (COSM 19524) | 25.1 | — | — |
| Case 4 | TPS3          | P72R     | 60.3          | TPS3 R337C (COSM 11071) | 18.0 | —             | —             |
| Case 5 | TPS3          | P72R     | 46.9          | KDR Q472H     | 46.9 | KIT G534C     | 46.3          | APC S1463fs | 42.5 | — | — |
| Case 6 | PDGFRa        | P567Q    | 100           | TPS3 V73W     | 72.6          | TPS3 P151S | 57.5 | KDR Q472H | 42.4 | ERBB4 C614Y | 38.2 |
| Case 7 | PTEN          | L57W (COSM 5253) | 21.2          | —             | —             | —             | —             |
| Case 8 | TPS3          | P72R     | 98.4          | KIT M541L (COSM 28026) | 98.4 | TPS3 V154G (COSM 43903) | 35.4 | KDR Q472H | 26.7 | SMAD4 G432R | 14.7 |
| Case 9 | ABL1          | I347fs   | 11.1          | ERBB4 C759T   | 8.8           | FBXW7 M467I | 8.0           | MLH1 A169V | 8.0 | KDR G1284R | 7.9 |
| Case 10 | APC P1433L    | 6.7       | TPS3 F338L   | 6.5           | SMO P630S    | 6.4           | MET D340A | 5.8 | NOTCH1 V1575M | 5.7 |
| Case 11 | PTEN A328E    | 5.6       | APC G1374K (COSM 18737) | 5.1 | MLH1 R148W | 5.0 | — | — | — | — |
| Case 12 | ABL1          | E1464fs  | 59.2          | TPS3 P72R     | 48.2          | BRAF G442D | 6.1           | MET G1102D | 5.5 | SMO T223I | 5.0 |
| Case 13 | MET N375K     | 55.7      | TPS3 P72R     | 42.0          | CNTN11 D352N | 15.1 | PTEN H123Y (COSM 5078) | 7.3 | PTEN R130Q (COSM 5033) | 7.2 | — | — |
| Case 14 | TPS3          | P72R     | 68.5          | PTEN N3209fs (COSM 4932) | 39.5 | TPS3 K132R (COSM 11582) | 29.7 | — | — | — | — |
| Case 15 | TPS3          | P72R     | 98.1          | KDR Q472H     | 96.4          | TPS3 V272fs | 21.0          | RB1 I682T | 12.6 | APC P1433L | 9.6 |
| Case 16 | RET E884V     | 9.1       | SMAD4 V354L  | 8.0           | —             | —             | —             | — | — | — | — |
| Case 17 | TP53          | P72R     | 99.1          | CDX2 W1555    | 51.6          | FLT3 W603* | 45.2          | KRAS E37K | 33.3 | SMO P641L | 23.7 |
| Case 18 | IDH1 L103M    | 20.0      | TPS3 R267Q (COSM 43923) | 18.8 | GNA11 D205N | 16.2          | SMARCB1 P1655 | 14.0 | RB1 M761T | 13.9 |
| Case 19 | SMARCB1 V145L | 12.4      | TPS3 Q245R (COSM 10957) | 10.8 | NOTCH1 H1591T | 10.7          | ERBB4 G240V | 10.0 | KIT S715N | 9.9 |
| Case 20 | FBXW7 R505H (COSM 25812) | 9.8 | FBXW7 M498L | 9.2 | MET S186L | 8.8 | IDH1 A111V | 8.8 | JAC3 V133I | 8.5 |
| Case 21 | KIT V825I (COSM 19110) | 8.1 | TPS3 G112S | 6.5 | TPS3 K132E (COSM 10813) | 6.3 | HNFA1 A193V | 6.3 | VHL K171T | 5.7 |
| Case 22 | ALK P1191A    | 5.6       | HNF1A T204I | 5.3           | —             | —             | —             | — | — | — | — |
| Case 23 | PTEN H1047L   | 62.9      | FGFR3 R765S  | 7.2           | IDH1 P118L | 5.7 | —             | — | — | — | — |
| Case 24 | PTEN          | P72R     | 100           | MET A179M    | 5.1           | —             | —             | — | — | — | — |
a potential role of the \textit{KIT} M541L variant in the sensitivity of Imatinib for aggressive fibromatosis \cite{44-46}. Furthermore, a wide array of in vitro analyses support a role for the L541 variant in tumorigenesis, FDC-P1 cells transfected with \textit{KIT}-L541 showed an enhanced proliferative response, while \textit{KIT}-L541 cells were more sensitive to imatinib than those expressing wild-type \textit{KIT} \cite{47}. Inokuchi, et al. observed a higher frequency of L541 variants among patients with chronic myelogenous leukemia (CML), which is consistent with increased tyrosine kinase activation and proliferative responses in \textit{KIT}-L541 cells relative to wild-type controls \cite{48}. From the view point of EGFR-TKI resistance, these data suggest a causative role for the \textit{KIT} L541 variant in recurrence and drug resistance of NSCLC. Suppression of KIT with drugs like Imatinib may be a useful therapeutic choice in patients with \textit{KIT}-variant tumors.

Five (cases 3, 4, 5, 12 and 13) out of six NSCLC patients that are negative for \textit{EGFR}-T790M mutation harbored “compound mutations” (a rare \textit{EGFR} mutation in combination with a more frequent activating mutation). On the other hand, all T790M-positive tumors (cases 1, 6, 7, 8, 9, 10 and 11) lack an additional rare mutation apart from the presence of a frequent inhibitor-sensitive \textit{EGFR} mutation. Among these compound mutations (specifically rare mutations), tumors harboring S768I in exon 20 is known as resistant to EGFR-TKIs. On the contrary, tumors harboring point mutations in exon 18 and dual mutation of exon 19 deletion and S768I are reported to possible response to EGFR-TKIs. There have been limited data in other compounds mutations. So a role of these mutations in causing drug resistance in T790M-negative patients is uncertain and need to be evaluated \cite{49}.

This study has its limitations. The strongest limitations include a small sample size, and the retrospective nature of the study preventing the comparison of our findings to non-lesional or pre-treatment results. With this limitation of not having pre-treatment results, the role of activating mutations in additional oncogenes in TKI-resistance may be the primary cause for TKI resistance especially in the case of KDR Q427H mutations. A larger prospective study with strict enrollment criteria is definitely needed to overcome these limitations.

### Conclusion

In conclusion, our study showed that NGS could be useful to detect \textit{EGFR} T790M variants in patients not otherwise found with other conventional PCR based methods. Furthermore, our results highlight the difference of the extent of \textit{EGFR} T790M and other \textit{EGFR}-activating mutations among tumor samples, which may indicate the heterogeneity of acquired mutations. Identification of additional sequence variations in potential oncogenes that may affect \textit{EGFR}-TKI resistance would suggest a series of new therapeutic agents targeting on a patient’s underlying genetic profile.

### Competing interests

The authors declare that they have no competing financial and non-financial interests.

### Authors’ contributions

KM and SF carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. MM gave us some technical information. KM and SF participated in the conception and design of the study and performed the statistical analysis. KM, SF, AH, CO, KO, RK, JT, RK, NK and YH engaged in the acquisition and interpretation of data. KM and SF was involved in drafting the manuscript. KM, SF participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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