Research paper

Next-generation sequencing reveals novel resistance mechanisms and molecular heterogeneity in EGFR-mutant non-small cell lung cancer with acquired resistance to EGFR-TKIs

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ARTICLE INFO

Keywords:
Epidermal growth factor receptor
Tyrosine kinase inhibitor
Non-small cell lung cancer
Resistance mechanism
Next-generation sequencing

ABSTRACT

Objectives: Despite initial responses to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in EGFR mutant non-small cell lung cancer, patients invariably develop acquired resistance. In this study, we performed next-generation sequencing in pre- and post-EGFR-TKI tumor samples to identify novel resistance mechanisms to EGFR-TKIs.

Material and methods: We collected tumor tissues before EGFR-TKI treatment and after progression from 19 NSCLC patients to analyze genomic alterations in 409 cancer related genes. Bioinformatics analyses were used to identify mutations in which the allele frequencies are significantly changed, or newly appeared after progression.

Results: Overall, mutation rates and compositions were similar between pre- and post-EGFR-TKI tumors. We identified EGFR T790M as the most common mechanism of acquired resistance (63.2%). No pre-EGFR-TKI tumor had a preexisting T790M mutation, suggesting that tumors acquired T790M mutations following progression on EGFR-TKIs. Compared to T790M-positive tumors, T790M-negative tumors showed relatively high tumor mutation burden and shorter survival, suggesting T790M-negative patients as a potential candidate for immune checkpoint inhibitors. TP53 mutation was also significantly enriched in the T790M-negative tumors. Finally, we described here for the first time a novel missense mutation (T263P), which occurred concurrently with an activating G719A mutation, in the extracellular domain II of EGFR in a patient with poor response to erlotinib.

Conclusion: Comprehensive genomic analysis of post-EGFR-TKI tumors can provide novel insight into the complex molecular mechanisms of acquired resistance to EGFR-TKIs.

1. Introduction

The mutations of tyrosine kinase domain of the epidermal growth factor receptor (EGFR) increase lung cancer’s sensitivity to EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib or afatinib. Despite initial response, most patients experience tumor progression due to acquired resistance to the treatment [1,2].

The substitution of threonine to methionine at codon 790 (T790M) of tyrosine kinase domain of EGFR contributes to the acquired resistance to EGFR-TKIs in approximately half of the patients who progressed on gefitinib or erlotinib. Other mechanisms such as MET amplification, phenotypic transformation and bypass tract activation have also been involved in the acquired resistance to EGFR-TKIs [3]. However, more research on the acquired resistance mechanisms will be
needed, because approximately 30% of cases still remain unknown [3].

The most effective way to identify genetic aberrations that cause acquired resistance is to directly compare the genomes between pre-EGFR-TKI and post-progression samples. Generally, traditional assays, such as Sanger sequencing or PCR-based assays, have been used to interrogate known hotspots in a small scale. In contrast, next-generation sequencing (NGS) enabled comprehensive genome-wide inspection of genetic aberrations to warrant clinical applications [4]. Most recently, NGS has been actively applied to paired analyses of a same patient between different time points across interventions [5,6]. However, the difficulties in collecting and securing the sufficient amount of intact tumor specimen for NGS analysis complicate a scaled analysis in a clinical setting that does not include a surgical resection.

In this study, we performed NGS in paired tumor samples before and after EGFR-TKIs to better understand and identify the novel resistance mechanisms to EGFR-TKIs.

2. Materials and methods

Detailed procedures are provided in the Supplementary Materials and Methods.

2.1. Study population

Patients with advanced EGFR mutant NSCLC who were treated with erlotinib or gefitinib, had pre-EGFR-TKI and/or post-progression samples available, and met Jackman’s criteria [7] regarding the clinical definition of acquired resistance were included. Progression-free survival (PFS) was defined as the time from the first administration of erlotinib/gefitinib to the time of radiologic confirm of progression, or of treatment cessation. The overall survival (OS) was defined as the time from first administration of EGFR-TKIs to the time of the patient death. The post-progression survival (PPS) was defined as the difference between OS and PFS. All patients provided written, informed consent before sample acquisition. This study was reviewed and approved by the Institutional Review Board of Severance Hospital, Seoul, Korea. This study was conducted in accordance with the Declaration of Helsinki.

2.2. Sample handling and sequencing

Formalin-fixed paraffin-embedded (FFPE) samples were reviewed by pathologists for DNA isolation and quality control. Genomic DNA from tumors and matching blood was prepared for sequencing of 409 cancer-related genes using Ion AmpliSeq Comprehensive Cancer Panel. The sequencing datasets generated during the current study are not publicly available since other data are on work to be published, but are available from the corresponding author on reasonable request.

2.3. Variant detection and annotation

Raw NGS reads were mapped to the hg19 human reference genome. Mapping and pro-processing procedures were optimized for analysis of Ion Torrent™ data, including duplicate removal and homopolymer handling [8]. The aligned NGS data were used to detect 1) somatic point mutations and 2) somatic indels. For somatic point mutations, initial call-sets of two somatic mutation callers MuTect [9] and Virmid [10] were merged and further filtered. Functional impacts of the mutations were annotated using Seattle-Seq [11] to extract only non-silent coding mutations. For somatic indels, we kept more conservative criteria in calling due to the known susceptibility of Ion Torrent™ around homopolymer regions. Initially, indels were called using GATK HaplotypeCaller [12] for tumor and normal separately, and ones that are only presented in tumor were selected. Additional filters regarding read depth, mapping quality, germ line removal, homopolymer and repeat genomic regions were applied. Indels within known hotspot sites are further manually inspected to rescue clinically important variants with a low variant allele frequency (see Supplementary Materials and Methods and Supplementary Fig. S1 for details).

Genes that are recurrently mutated in the post-progression tumor (≥1 samples) were selected based on the somatic mutation call-sets. The presence of mutations in the pre-treatment sample was judged based on the number of alternative variant alleles at the site. Each recurrent mutation was annotated with its functional impact on cancer [13–15].

2.4. In silico functional analysis

The effect of the mutation on the protein conformational change was predicted using SWISS-MODEL and RAMPAGE. PyMOL (The PyMOL Molecular Graphics System, DeLano Scientific LLC, USA) was used for molecular visualization of the mutant structure model.

3. Results

3.1. Patient characteristics

Nineteen patients satisfied the clinical definition of acquired resistance to EGFR-TKIs (Table 1). The median age was 58 years (range: 36–72 years). The majority of patients were women (n = 11, 63.2%). Most patients were never smokers (n = 11, 57.9%). All of the patients were treated with erlotinib (n = 2) or gefitinib (n = 17). Fourteen patients (73.7%) had exon 19 deletions. EGFR mutation was not detected in one patient (Patient 7). MET amplification by FISH analyses, or small cell lung cancer transformation by histologic examination was not detected in any of post-progression samples. The median PFS to EGFR-TKIs was 6.7 months (range: 2.4–27.8). The best overall response to EGFR-TKIs was a partial response in 10 patients (52.6%), and stable disease in 8 (42.1%).

3.2. Genomic landscape of post-progression tumors

Tumors were sequenced to a median coverage of 607x. A total of 1398 somatic single-nucleotide variants (SNVs) and 1774 indels were detected from the 19 patients (Fig. 1, Supplementary Fig. S1, and S2). The number of SNVs and indels varied widely across patients (1–561.43 SNVs and 0–215.99 indels per Mb). The total number of somatic mutations was similar between pre-EGFR-TKI and post-progression samples. Notably, one patient (P3) had an extremely high number of mutations. The number of non-silent SNVs was higher (1–397.45 per Mb) than that reported in previous studies (3–16 per Mb) [16,17]. The increased number of mutations may be explained by 1) the high sequencing depth that enables detection of low-allele frequency mutations, 2) the lenient calling criteria designated for a novel mutation discovery; and 3) the intrinsically high error rate of the Ion Torrent™ sequencing platform, which is inevitable in biopsy sample analysis. Therefore, the mutation landscape in this study should be interpreted to include a certain level of putative variants. After resistance acquisition, there were no notable changes in the mutation types and the transition to transversion ratio (Ti/Tv). Overall, the similar mutation rates and compositions from pre-EGFR-TKI and post-progression tumors that acquired resistance is likely governed by specific gene mutations.

3.3. Analysis of genetic alterations in progressed lung cancer tumor

In 17 patients, we analyzed significantly mutated genes in post-progression samples compared to pre-EGFR-TKI samples (excluding P5 and P7, whose samples lacking paired pre-EGFR-TKI samples or blood samples). We identified genes that were significantly mutated as well as genes with putative functional oncogenic significance (Fig. 2, Supplementary Table S1). Two T790M-negative tumors (P1 and P3) with extremely low PFS (2.4 and 3.9 months) had high numbers of significantly
mutated genes in their post-progression samples (24 and 17 mutated genes, respectively). When we analyzed the significantly mutated genes using MuSiC [18], TP53 was the only gene that was significantly mutated in the T790M-negative tumors (Supplementary Table S2).

### 3.4. Comparison of EGFR mutant allele frequencies between pre-EGFR-TKI and post-progression samples

We next compared the allele frequency (AF) of sensitizing EGFR mutations (exon 19 deletion and L858R) and T790M in pre-EGFR-TKI and post-progression samples (Fig. 3 and Supplementary Table S3). The AFs of exon 19 deletion and L858R sensitizing mutations were increased along with T790M mutation, except for 4 cases (P2, P12, P15, P17). In P6, AF of exon 19 deletion mutation was dramatically increased from 17% to 57% in the absence of T790M mutation, suggesting outgrowth of resistant clones harboring non-T790M mutations (Fig. 3C). Consistent with MTT assays, Ba/F3 cells harboring G719S/T263P mutation showed significantly higher sensitivity to afatinib versus gefitinib (IC50 value, 0.002 nM versus 119.7 nM) (Fig. 5F). Since our model suggest that co-occurrence of T263P mutation with sensitizing EGFR mutation may contribute to de novo resistance by enhancing EGFR-HER2 heterodimerization, we hypothesized that T263P occurred in the extracellular domain II of the EGFR immediately before the extended loop that contacts domain IV (Fig. 5D). Even in the absence of EGF ligand, this mutation allows the EGFR extracellular domains to be extended, or in open conformation for dimerization, leading to de novo resistance to selective EGFR-TKIs, such as afatinib or erlotinib (Fig. 5E). In one case (P4), we identified a novel missense mutation (threonine-to-proline substitution at amino acid 263; T263P) of the extracellular domain (subdomain II) of EGFR, concurrently with an EGFR activating G719A mutation in both samples (Fig. 5A, B). This patient had a poor response to erlotinib (PFS = 3.9 months). We confirmed the presence of EGFR T263P/G719A mutation with Illumina™ Amplicon sequencing (Fig. 5C). The EGFR T263P mutation was reported to be oncogenic in glioblastoma [19]. However, this novel mutation has not been reported in NSCLC. Using in silico 3D modeling, we hypothesized that T263P occurred in the extracellular domain II of the EGFR immediately before the extended loop that contacts domain IV (Fig. 5D). Even in the absence of EGF ligand, this mutation allows the EGFR extracellular domains to be extended, or in open conformation for dimerization, leading to de novo resistance to selective EGFR-TKIs, such as afatinib or erlotinib (Fig. 5E). Since our model suggest that co-occurrence of T263P mutation with sensitizing EGFR mutation may contribute to de novo resistance by enhancing EGFR-HER2 heterodimerization, we tested whether pan-HER inhibitors such as afatinib may be more active by blocking heterodimerization of the open form of EGFR with HER2 in this co-mutant cells than gefitinib. We compared the cytotoxicity of gefitinib with pan-HER inhibitor afatinib, against Ba/F3 cells harboring EGFR G719S mutation concurrently with or without T263P mutation. Strikingly, Ba/F3 cells harboring G719S/T263P mutation showed significantly higher sensitivity to afatinib compared to gefitinib (IC50 value, 0.002 versus 119.7 nM), whereas Ba/F3 cell harboring only G719S mutation showed sensitivity to both afatinib and gefitinib (IC50 value, 0.003 versus 7.256 nM) (Fig. 5F). Consistent with MTT assays, Ba/F3 cells harboring G719S/T263P mutation showed significant suppression of p-EGFR and p-ERK upon treatment with afatinib, whereas Ba/F3 cells harboring only G719S mutation showed potent inhibition of p-EGFR and p-ERK by either gefitinib or afatinib (Fig. 5G). Taken together, these in vitro results support our hypothesis that EGFR T263P mutation lead to heterodimerization of EGFR with HER2,

### Table 1
Baseline clinical patient characteristics.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender/age</th>
<th>Clinical stage at diagnosis</th>
<th>Smoking status at diagnosis</th>
<th>TKI</th>
<th>TKI line</th>
<th>Brain Metastasis</th>
<th>PFS</th>
<th>Best response</th>
<th>EGFR mutation</th>
<th>MET amplification</th>
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<td>IV</td>
<td>Never smoker</td>
<td>Gefitinib</td>
<td>Palliative 1st</td>
<td>No</td>
<td>2.4</td>
<td>PD</td>
<td>Exon 19 Del</td>
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<td>Never smoker</td>
<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>Yes</td>
<td>3.6</td>
<td>SD</td>
<td>L858R</td>
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<tr>
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<td>IIIA</td>
<td>Never smoker</td>
<td>Gefitinib</td>
<td>Palliative 4th</td>
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<td>3.9</td>
<td>PR</td>
<td>Exon 19 Del</td>
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<tr>
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<td>Ex-smoker</td>
<td>Erlotinib</td>
<td>Palliative 2nd</td>
<td>Yes</td>
<td>3.9</td>
<td>SD</td>
<td>G719A</td>
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<td>Never smoker</td>
<td>Gefitinib</td>
<td>Palliative 1st</td>
<td>Yes</td>
<td>4.5</td>
<td>PR</td>
<td>Exon 19 Del</td>
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</tr>
<tr>
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<td>M/52</td>
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<td>Gefitinib</td>
<td>Palliative 3rd</td>
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<td>5.6</td>
<td>PR</td>
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</tr>
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<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>No</td>
<td>6.6</td>
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</tr>
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<td>IIIA</td>
<td>Current Smoker</td>
<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>No</td>
<td>5.4</td>
<td>PR</td>
<td>Exon 19 Del</td>
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<td>Palliative 1st</td>
<td>No</td>
<td>5.7</td>
<td>PR</td>
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<td>Yes</td>
<td>6.7</td>
<td>SD</td>
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<td>7.5</td>
<td>PR</td>
<td>Exon 19 Del</td>
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<td>Gefitinib</td>
<td>Palliative 1st</td>
<td>No</td>
<td>8.5</td>
<td>SD</td>
<td>L858R</td>
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<td>Never smoker</td>
<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>No</td>
<td>12.4</td>
<td>PR</td>
<td>L858R</td>
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<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>Yes</td>
<td>12.4</td>
<td>SD</td>
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<td>Gefitinib</td>
<td>Palliative 2nd</td>
<td>Yes</td>
<td>13.5</td>
<td>PR</td>
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<td>Palliative 2nd</td>
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<td>19.1</td>
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<tr>
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<td>Gefitinib</td>
<td>Palliative 1st</td>
<td>No</td>
<td>22.4</td>
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<td>Exon 19 Del</td>
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<tr>
<td>P19</td>
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<td>IV</td>
<td>Never smoker</td>
<td>Erlotinib</td>
<td>Palliative 3rd</td>
<td>No</td>
<td>27.8</td>
<td>PR</td>
<td>Exon 19 Del</td>
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</table>

*Patient without pre-EGFR TKI sample. §Patient without matched blood sample.
thereby activating downstream signaling pathway, in the presence of a reversible first-generation EGFR-TKIs, such as gefitinib or erlotinib, selective for EGFR. Our results also suggest potential role of the irreversible second-generation pan-HER TKI, such as afatinib, in patients with activating EGFR mutation concurrently with T263P.

4. Discussion

Our study is among the first to report the NGS results of paired tumor samples before and after treatment with EGFR inhibitors in patients who acquired resistance. Our results revealed a novel resistance mechanism of EGFR extracellular domain mutation. In addition, our data demonstrate molecular heterogeneity in EGFR mutant NSCLC cases with acquired resistance to EGFR-TKIs.

NGS sequencing and relevant analysis pipelines are now well established across multiple types of samples. However, in a technical aspect, accurate variant analysis using archived FFPE samples still considered difficult, thereby remaining a challenge to this study. Moreover, only a limited volume of sample could be collected from each patient by needle biopsy, narrowing down the number of choices.
for sequencing platforms such as Illumina. The Ion AmpliSeq Cancer Panel, among the remaining options, was selected for this study, in light of the previous benchmark studies for clinical cancer variant analysis [20]. We applied the specialized analysis pipelines for variant calling in Ion Torrent sequencing data including additional strict filters and a manual inspection procedure. As a result, we could show the number of total mutation is compatible with previous studies [21], while reporting an increased number of small indels [22–24]. As the techniques for

Fig. 2. Genes that were significantly mutated only in post-progression samples. Asterisk (*) of a gene indicates that the VEST score is significant (p-value ≤ 0.05). Asterisk (*) in block means that the mutation has COSMIC ID.

Fig. 3. Allele frequency change of EGFR exon 19 deletion (A), L858R (B), and T790M (C) between pre-EGFR-TKI and post-progression samples are shown.
sample and library preparation are improving as well as the throughput in the NGS technologies, we expect that a continuing investigation with a future low-volume, FFPE compatible sequencing will add more validity to our designed study.

Our survival analyses demonstrated that patients with the acquired T790M mutation had significantly better mPFS and mOS than did those without the T790M mutation. Several studies have reported that both Asian and Caucasian patients with T790M mutation positive lung cancer have better prognosis with regard to PPS (but not the case in PFS or OS) than did those without this mutation [25,26]. These observations are supported by the preclinical finding that T790M mutated lung cancer cells have more indolent growth compared to that of parental cells [27]. The unique PFS benefit among T790M positive patients in this study might be derived from undetected tumor heterogeneity in previous studies, since previous studies showing a PFS benefit for T790M positive patients did not perform deep sequencing. However, these patients might have had T790M positive minor clones. There is no study that compares patients with pre-existing minor T790M positive mutations to those with the newly acquired T790M; however, some studies have reported that patients with the pre-existing minor population of the T790M mutation were unresponsive to EGFR-TKIs [28]. Through deep sequencing, we were able to show that every T790M mutation in the post-progression samples was newly acquired, and not from proliferation of T790M positive minor clones. Although we cannot exclude pre-existing T790M-positive cancer cells in pre-EGFR-TKI sample due to sensitivity of NGS, we could conclude that tumors develop de novo gatekeeper T790M mutations in cancer cells at the time of progression. This may have contributed to our observation of better PFS among the T790M positive patients. There may be other mechanisms of resistance in patients without the T790M mutation with short PFS [29]. One potential mechanism is the TP53 mutation, which is significantly enriched among T790M-negative patients in MuSiC analysis. Another potential mechanism might be the consequences of high tumor mutation burden (TMB). In this study, P3 with extremely large number of mutations without T790M mutation, had very short PFS. Altogether, higher TMB or TP53 mutation may be attributed to genomic instability in T790M-negative patients, leading to short survival [30]. Recently, higher TMB has been reported as a predictor for sensitivity to immune checkpoint inhibitor [31,32]. Our result suggest that T790M-negative EGFR mutant NSCLC patients after progression on EGFR-TKIs may be considered as potential candidate for immune checkpoint inhibitors, as shown in one recent study [33]. This hypothesis is being tested in the CheckMate 722 study (NCT02864251). Collectively, these inter-tumoral heterogeneity shown in our study may have impact on patient survivals, as was shown in previous reports [34]. The conceptual schemes showing the suggested mechanisms of acquired resistance to EGFR-TKIs are depicted in Fig. 6. Since this study had relatively small sample size and their heterogeneity with regard to treatment lines and metastasis status, which might led to the comparatively shorter PFS of total population than reported in the literature [35,36], validation study of our concept with larger patients is needed.

In this study, we have shown novel extracellular-domain missense EGFR mutations in lung cancer patients that may be related to an inadequate response to EGFR-TKIs. Some mutations in extracellular domains II and IV of EGFR disrupt the closed conformation of EGFR, and make the receptor assume an “active” conformation in the absence of ligand binding [37]. Our study identified a novel missense mutation (T263P) of the extracellular domain (domain II) of EGFR, for the first time in lung cancer. In a glioblastoma model, the extracellular domain EGFR mutants selectively responded to type-II EGFR kinase inhibitors like lapatinib but not to first generation EGFR kinase inhibitors, probably due to differences in the binding properties of EGFR inhibitors [38]. We believe that the lung cancer bearing extracellular domain T263P mutant will also respond to type II EGFR kinase inhibitors like lapatinib. In addition, because the T263P mutation is thought to induce the extracellular EGFR domains into the opened form, making it easier to heterodimerize with HER2 receptors (see Fig. 5E), efficient treatment results with EGFR and HER2 dual-inhibitors like afatinib make sense. Our in vitro validation experiments showed that cells with EGFR T263P mutation were only sensitive to afatinib not to gefitinib. Also, our western blot analyses results added indirect evidence toward the molecular mechanism of T263P mutation leading to resistance to the first generation EGFR-TKIs – heterodimerization of T263P mutant EGFR with HER2 and downstream signals activation regardless of kinase domain inhibition. Therefore, these results revealed a novel mutation that is a drug target for this subset of patients.

In conclusion, our study demonstrates that NGS of pre-EGFR-TKI and post-progression tumor samples provides insight into the complex molecular mechanisms of acquired resistance to EGFR-TKIs in EGFR-mutant NSCLC. Patients who newly acquire the T790M mutation during treatment with EGFR-TKI have better survival than do those without it. Further investigations are warranted to develop optimal treatment strategies for these patients. Furthermore, a prospective pilot clinical study is needed to test EGFR and HER2 inhibitors, such as afatinib, for the small subset of patients with T263P EGFR mutations.
Fig. 5. Identification of a de novo EGFR extracellular domain II mutation in a patient with a poor response (PFS 3.9 months) to treatment despite a sensitive mutation (G719A). (A) Change of allele frequencies of EGFR exon 18 sensitive mutation G719A and newly identified EGFR extracellular domain II mutation T263P in pre-EGFR-TKI and post progression samples from patient P4. (B) Visualization of the identified novel T263P mutation, inspected via the Ion Torrent™ platform. (C) Validation of T263P mutation with Illumina™ TruSeq Amplicon sequencing with accurate variant allele frequency in A. (D) The domain organizations of EGFR and 3D structure of wild type EGFR extracellular domains are presented. Thr263 is also annotated. (E) Schematic model of EGFR T263P mutation and its role in EGFR dimerization and pathway activation. (F) Dose dependent growth inhibition of engineered G719S mutant Ba/F3 cell line with or without T263P mutation, by gefitinib or afatinib for 72 h is shown; bars, S.D. The determined IC50 value is also presented. (G) EGFR mutant Ba/F3 cells were treated with 1 μM of Gefitinib or Afatinib for 2 h, lysates were immunoblotted for phospho-EGFR (Tyr1068) and phospho-ERK (Thr202/Tyr204).

Abbreviations: TM, transmembrane segment; TK, tyrosine kinase domain; C-tail, c-terminal tail (auto-phosphorylation domain)
Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This research was supported in part by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2015R1A1A1A01053638, 2016R1A2B3016282); and by the National Research Foundation of Korea (NRF) grant funded by the Korea government(MSIP) (2015R1A2A1A15055817). Sangwoo Kim was supported by a faculty grant of Yonsei University College of Medicine (6-2016-0081).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2017.09.005.

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