Heterogeneity in resistance mechanisms causes shorter duration of epidermal growth factor receptor kinase inhibitor treatment in lung cancer

Kenichi Suda a,⁎, Isao Murakami b, Kazuko Sakai c, Kenji Tomizawa a, Hiroshi Mizuuchi a, Katsuaki Sato a, Kazuto Nishio c, Tetsuya Mitsudomi a

a Division of Thoracic Surgery, Department of Surgery, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Japan
b Department of Respiratory Medicine, Higashi-Hiroshima Medical Center, 513 Jike, Saio-Cho, Higashi-Hiroshima, Japan
c Department of Genome Biology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Japan

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Objectives: Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are used as a first line therapy for metastatic lung cancer harboring somatic EGFR mutation. However, acquisition of resistance to these drugs is almost inevitable. T790M (threonine to methionine substitution at codon 790 of the EGFR gene) and MET amplification are well-known resistance mechanisms, and we previously demonstrated that three of six autopsied patients showed inter-tumor heterogeneity in resistance mechanisms by analyzing T790M and MET gene copy numbers (Suda et al., 2010). To further elucidate the role of heterogeneity in acquired resistance, here we performed further analyses including additional five patients.

Materials and methods: We analyzed somatic mutations in 50 cancer-related genes for 26 EGFR-TKI refractory lesions from four autopsied patients using target sequencing. MET and ERBB2 copy numbers were analyzed by real-time PCR. Data for additional one patient was obtained from our recent study (Suda et al., 2015). Relationship between heterogeneity in resistance mechanism(s) and time to treatment failure (TTF) of EGFR-TKI and post-progression survival (PPS) were analyzed.

Results and conclusion: We observed heterogeneity of resistance mechanisms in two of four patients analyzed (T790M + MET gene copy number gain, and mutant EGFR loss × unknown). We also identified quantitative heterogeneity in EGFR T790M mutation ratio among EGFR-TKI refractory lesions. In analyzing patient outcomes, we found that patients who developed multiple resistance mechanisms had shorter TTF compared with those who developed single resistance mechanism (p = 0.022). PPS after EGFR-TKI treatment failure was compatible between these two groups (p = 0.42). These findings further our understanding of acquired resistance mechanisms to EGFR-TKIs, and may lead to better treatment strategies after acquisition of resistance to first generation EGFR-TKIs in lung cancer patients with EGFR mutations.

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1. Introduction

Lung cancers with somatic epidermal growth factor receptor (EGFR) gene mutations account for ~40% of lung adenocarcinomas in East Asians and ~15% of those in Caucasians and African Americans. Based on recent phase III trials [1–6], EGFR tyrosine kinase inhibitors (TKIs) are usually administered as a front-line therapy for patients with metastatic lung cancer harboring EGFR mutation. However, despite initial dramatic responses to these drugs, the emergence of resistance is almost inevitable after a median of approximately 1 year. So far, several resistance mechanisms have been identified [7–9]. These include the so-called gate-keeper mutation of the EGFR gene (T790M mutation), MET gene amplification, ERBB2 gene amplification, MAPK gene amplification, PIK3CA mutations, BRAF mutations, AXL activation, and transformation to small cell lung cancer.

For patients with acquired EGFR T790M secondary mutation, T790M specific EGFR-TKIs, so-called third generation TKIs, have shown remarkable treatment efficacy in clinical trial settings [10,11]. In addition, several case studies have reported the effec-

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tiveness of cytotoxic chemotherapy for small cell lung cancer in patients whose tumors transformed to small cell lung cancer after acquisition of resistance to EGFR-TKIs [7]. Therefore, re-biopsy for EGFR-TKI refractory lesions and analysis of the resistance mechanisms may provide clinical benefit for patients who acquired resistance to EGFR-TKIs.

On the other hand, recent studies have shown that lung cancers acquire considerable spatial as well as temporal heterogeneities during their development [12,13]. The heterogeneity is further exaggerated by the presence of pharmacologic intervention. Therefore, understanding the potential heterogeneity of resistance mechanisms that would directly relate to patient outcome is critical. Our previous analysis for EGFR T790M mutation and MET gene copy number using tumor samples from six autopsied patients demonstrated that three patients showed inter-tumor heterogeneity in resistance mechanisms [14]. To further extend this observation, in this study, we examined an additional four autopsied patients using next generation sequencing technology that enabled us to search for amplification and mutations of 50 cancer-related genes.

2. Materials and methods

2.1. Patient characteristics

Our cohort consisted of four autopsied patients with lung adenocarcinoma who developed multiple refractory tumors to first-generation EGFR-TKI (Cases 1–4 in Table 1). Genomic DNA (gDNA) was extracted from each TKI-refractory lesion (26 lesions in total) as previously described [15]. In Cases 1–3, gDNA at the time of initial lung cancer diagnosis (pre-treatment) was available for analysis. Approval from the institutional review board for use of these tumor specimens was obtained from the patients’ legal guardians.

Clinical and molecular data of an additional 7 autopsied patients that we reported previously [14,16] were also used in this study to evaluate the correlation between heterogeneity of resistance mechanisms and patient outcomes.

All 11 patients harbored EGFR mutations that were linked to drug sensitivity, had initially responded to EGFR-TKI monotherapy (complete/partial response or stable disease lasting more than 6 months), and then developed resistance while on continuous treatment with the drug. No patients received second- or third-generation EGFR-TKIs. Approval for the use of tumor specimens for these analyses was obtained from the institutional review boards of Kinki University Faculty of Medicine and Higashi-Hiroshima Medical Center.

2.2. Target sequencing analysis

Target sequencing analysis for 50 cancer-related genes was performed as previously described [17]. Briefly, we used 10 ng of gDNA for the multiplex PCR amplification using the Ion AmpliSeq Library kit 2.0 (Life Technologies, Carlsbad, CA) and the Ion AmpliSeq Cancer Hotspot panel v2 (Life Technologies). The Ion Xpress Barcode Adapters (Life Technologies) were ligated into the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The purified libraries were then pooled and sequenced on an Ion Torrent PGM device using the Ion PGM 200 Sequencing kit v2 and the Ion 318 v2 Chip kit (all from Life Technologies). DNA sequencing data were accessed through the Torrent Suite v4.2 software program. Reads were aligned against the hg19 human reference genome, and variants were called using the variant caller v4.2. Raw variant calls were filtered out using the following annotations: homozygous and heterozygous variants, quality score of <100, and depth of coverage <19. Germline mutations were excluded using the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SpnDB).

For the analysis of T790M ratio, which is defined as allele frequency of M790 relative to EGFR allele with activating mutation, allele count data for EGFR activating mutations (L858R or exon 19 deletion mutation) and those for EGFR T790M mutation were used. The T790M ratio was calculated as allele count of T790M divided by that of EGFR activating mutation. Because allele count data includes sequencing errors, for tumors with lower T790M allele count data compared with the average +3 standard deviation of that of 10 normal gDNA, T790M mutation status was assessed as negative.

2.3. Gene copy number analyses

MET gene copy numbers and ERBB2 gene copy numbers relative to a LINE-1 repetitive element were measured by quantitative real-time PCR using the SYBR Green Method (Power SYBR Green PCR Master Mix; Qiagen) with a StepOnePlus system (Life Technologies) as previously described [14]. Primer sequences for MET and LINE-1 genes were the same as in our previous report [18]. Primers for ERBB2 were as follows: 5′-cagaagctctacagtggtctt-3′ (forward) and 5′-cactcctcaggtagctt-3′ (reverse). We performed PCR in triplicate for each primer set. LINE-1 was used as the internal control because LINE-1 copy numbers are reportedly similar in normal and cancerous cells [19]. Normal gDNA was used as a standard sample, and copy numbers >4 and >8 were defined as copy number gain (CNG) and gene amplification, respectively, following the previous report [14].

2.4. Statistical analyses

Correlations among variables were analyzed using Pearson’s rank test. Time to treatment failure (TTF) was defined as the time from initiation of EGFR-TKI therapy to the time of disease progression or to the time of drug discontinuation by any cause or death. Post-progression survival (PPS) was defined as the time from EGFR-TKI treatment failure to death. Differences in TTFs and PPSs of the two groups were compared with the Kaplan–Meier method and Log-rank test. Statistical analyses were performed with StatView version 5.01 (SAS Institute).

3. Results

3.1. Qualitative heterogeneity of resistance mechanisms

Among four patients with acquired resistance to EGFR-TKIs, we observed qualitative heterogeneity in acquired resistance mechanisms in two patients (Cases 2 and 3). Case 2 developed T790M secondary mutation in the para-aortic and para-pancreatic lymph nodes, omentum nodule, thyroid metastasis, and disseminations from right and left pleural spaces, but not in the primary tumor, liver metastases of right and left lobes, and pancreatic metastasis. Allele counts for the MET gene of the latter four lesions suggested increased copy number of MET, and this was confirmed as MET gene CNG by real-time PCR analysis (Fig. 1A).

Correlation between the allele counts for the MET gene detected at target sequencing and the copy numbers of MET gene identified by real-time PCR were evaluated using Pearson’s correlation coefficient. As shown in Fig. 1B, these two variables were significantly correlated ($R^2 = 0.68, P < 0.0001$).

In Case 3, pre-treatment gDNA obtained by trans-bronchial lung biopsy harbored L858R activating EGFR mutation and TP53 Y163H mutation. At autopsy, the primary lesion retained EGFR L858R and TP53 mutation without additional mutation (resistance mechanism
Table 1

Summary of autopsy cases in the present study and previous analyses.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Pack-year</th>
<th>Resistance mechanism</th>
<th>TTF (month)</th>
<th>PPS (month)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>57/F</td>
<td>0</td>
<td>T790M/MET amplification</td>
<td>13.8</td>
<td>9.9</td>
<td>[14]</td>
</tr>
<tr>
<td>CCR2</td>
<td>48/F</td>
<td>0</td>
<td>T790M/MET amplification</td>
<td>11.0</td>
<td>1.2</td>
<td>[14]</td>
</tr>
<tr>
<td>CCR3</td>
<td>58/M</td>
<td>34</td>
<td>MET amplification</td>
<td>14.5</td>
<td>6.7</td>
<td>[14]</td>
</tr>
<tr>
<td>CCR4</td>
<td>75/M</td>
<td>0</td>
<td>T790M</td>
<td>43.9</td>
<td>7.3</td>
<td>[14]</td>
</tr>
<tr>
<td>CCR5</td>
<td>93/F</td>
<td>0</td>
<td>T790M</td>
<td>14.8</td>
<td>16.1</td>
<td>[14]</td>
</tr>
<tr>
<td>CCR6</td>
<td>62/M</td>
<td>26</td>
<td>T790M/Unknown</td>
<td>9.1</td>
<td>10.0</td>
<td>[14]</td>
</tr>
<tr>
<td>Case 1</td>
<td>86/F</td>
<td>0</td>
<td>T790M</td>
<td>10.8</td>
<td>2.6</td>
<td>–</td>
</tr>
<tr>
<td>Case 2</td>
<td>72/M</td>
<td>27</td>
<td>T790M/MET amplification</td>
<td>3.8</td>
<td>2.8</td>
<td>–</td>
</tr>
<tr>
<td>Case 3</td>
<td>89/F</td>
<td>0</td>
<td>EGFR Loss/Unknown</td>
<td>9.0</td>
<td>0.2</td>
<td>–</td>
</tr>
<tr>
<td>Case 4</td>
<td>84/F</td>
<td>0</td>
<td>Unknown</td>
<td>22.6</td>
<td>4.5</td>
<td>–</td>
</tr>
<tr>
<td>SREP1</td>
<td>76/F</td>
<td>0</td>
<td>SCLC transformation/T790M</td>
<td>5.0</td>
<td>3.0</td>
<td>[16]</td>
</tr>
</tbody>
</table>

* TTF: time to treatment failure.
* PPS: post-progression survival.
* SCLC: small cell lung cancer.

Fig. 1. Quantitative and qualitative heterogeneity in acquired resistance mechanisms to EGFR-TKIs. (A) T790M ratio of each metastatic lesion of Case 1 (left) and Case 2 (right) are shown. “MET” in the figure indicates MET gene copy number gain (>four copies) identified by real-time PCR technique. (B) Correlation of the allele counts of target sequencing and copy numbers identified by real-time PCR technique in MET gene. (C and D) Comparison of time to treatment failure for EGFR-TKI treatment (C) and post-progression survival (D) between patients with single resistance mechanism versus those with multiple resistance mechanisms.
unknown), whereas metastatic liver tumor lost EGFR activating mutation but retained TPS3 mutation.

In Case 1, all EGFR-TKI refractory lesions but not pre-treatment sample harbored EGFR T790M mutation (Fig. 1A). Conversely, in Case 4, target sequencing analyses detected no candidate genetic aberration in all samples. All EGFR-TKI refractory lesions harbored EGFR E709C mutation together with L858R mutation (resistance mechanism unknown). ERBB2 CNG was not detected among these four patients (data not shown).

3.2. Quantitative heterogeneity of T790M mutation

Next, we focused our attention on the heterogeneity in the T790M ratio. As shown in Fig. 1A, the T790M ratio varied from 1.7% to 51.3% in Case 2, and 8.4% to 61% in Case 1. There was no obvious correlation between the T790M ratio and organs with metastatic tumors; however we noted that the T790M ratio was higher in all of the pleural disseminations.

3.3. Qualitative heterogeneity of resistance mechanisms and treatment efficacy

We also evaluated the role of the heterogeneity of resistance mechanism(s) on the patient outcomes including the data from our previous analyses [14,16]. Basic clinical characteristics, acquired resistance mechanism(s) and TTFs and PPSs after EGFR-TKI treatment failure are summarized in Table 1. One patient (Case 4 in this study) was excluded from the analysis for these survivals, because we could not identify the resistance mechanism. As shown in Fig. 1C, we observed shorter TTF in patients who developed qualitative heterogeneity in acquired resistance mechanisms (P=0.022). While, PPSs after EGFR-TKI treatment failure was compatible between these two groups (P=0.42, Fig. 1D).

4. Discussion

In this study, we observed qualitative heterogeneity of acquired resistance mechanisms to EGFR-TKIs in six of ten lung cancer patients with EGFR mutation in total (for one patient, we could not identify the resistance mechanism). Although this heterogeneity has been previously reported by our group [14] as well as others [20–22], here we firstly noted the possibility that the heterogeneity in resistance mechanism might be related to the shorter TTF. This observation can be interpreted as follows: if cancer cells possess an ability to develop multiple resistance mechanisms before treatment, these cells may have higher adaptability to pharmacologic insult, resulting in earlier emergence of resistance. However, we were only able to analyze 10 patients and we did not perform multi-variante analysis including factors that may also affect TTF; therefore more data are needed for final conclusion.

Secondly, we observed significant heterogeneity of T790M ratio among different refractory lesions. This quantification was made possible by the advent of next generation sequencing technology, eliminating the effect of contaminating normal cells. We have to keep in mind this heterogeneity, especially when we use irreversible EGFR-TKIs, so-called second generation EGFR-TKIs, because amplification of the T790M allele, which means high T790M ratio, has been reported to cause resistance to afatinib [23].

Using data of allele frequencies from target sequencing, we showed that the MET gene CNG can be well predicted (Fig. 2A; R² = 0.68). Therefore, we consider that target sequencing is appropriate for the analysis of re-biopsy samples, detecting not only mutations but also gene CNGs that are related to acquired resistance to EGFR-TKIs.

We were not able to detect any mutations in cancer-related genes in addition to the well-known “major” acquired resistance molecular mechanisms. For example, no PDK3CA mutation or STK11 mutation occurred concurrently together with EGFR T790M secondary mutation. These data would suggest the relatively simple and less heterogeneous molecular change(s) upon acquisition of resistance to EGFR-TKIs. Results of whole-genome/exome sequencing for acquired resistant in vitro models by Jia et al. compared with isogenic parental cells, support our observation; the only non-silent single nucleotide variants/indels identified was EGFR T790M mutation that impacted the three key phosphorylation residues (serine, threonine, and tyrosine) in the kinase domain of kinase genes [24]. Clinical high efficacy of T790M specific EGFR-TKIs also support this hypothesis [10,11].

In conclusion, we observed qualitative heterogeneity of acquired resistance mechanisms and/or quantitative heterogeneity of EGFR T790M mutation ratio between EGFR-TKI refractory lesions within a single patient. We observed that patients who developed multiple resistance mechanisms showed relatively shorter TTF compared with those who developed single resistance mechanism, although this result should be further discussed with more data. Understanding acquired resistance mechanisms, including tumor heterogeneity, would be essential for establishing better treatment strategies for lung cancer patients with EGFR mutations.

Conflict of interest

T. Mitsuodmi has received honoraria from AstraZeneca, Chugai, Boehlinger-ingelheim, Pfizer, Roche, Novartis, and Taiho; has played advisory roles for AstraZeneca, Chugai, Boehlinger-ingelheim, Pfizer, Roche, and Clovis Oncology; and has received research funding from AstraZeneca and Chugai. All other authors declare that they have no conflict of interest related to this study.

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