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

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ABSTRACT

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 number (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.

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/SnpDB).

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′-cagaaggtctacatgggtgctt-3′ (forward) and 5′-cacatcctccaggtagctcat-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