Sensitivities to various epidermal growth factor receptor-tyrosine kinase inhibitors of uncommon epidermal growth factor receptor mutations L861Q and S768I: What is the optimal epidermal growth factor receptor-tyrosine kinase inhibitor?

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Most patients with non-small cell lung cancer (NSCLC) harboring common epidermal growth factor receptor (EGFR) mutations, such as deletions in exon 19 or the L858R mutation in exon 21, respond dramatically to EGFR tyrosine kinase inhibitors (EGFR-TKI), and their sensitivities to various EGFR-TKI have been well characterized. Our previous article showed the in vitro sensitivities of EGFR exon 18 mutations to EGFR-TKI, but little information regarding the sensitivities of other uncommon EGFR mutations is available. First, stable transfectant Ba/F3 cell lines harboring EGFR L858R (Ba/F3-L858R), L861Q (Ba/F3-L861Q) or S768I (Ba/F3-S768I) mutations were created and their drug sensitivities to various EGFR-TKI were examined. Both the Ba/F3-L861Q and Ba/F3-S768I cell lines were less sensitive to erlotinib, compared with the Ba/F3-L858R cell line, but their sensitivities to afatinib were similar to that of the Ba/F3-L858R cell line. The Ba/F3-L861Q cell line was similarly sensitive and the Ba/F3-S768I cell line was less sensitive to osimertinib, compared with the Ba/F3-L858R cell line. The results of western blot analyses were consistent with these sensitivities. Next, similar experiments were also performed using the KYSE270 (L861Q) and KYSE 450 (S768I) cell lines, and their results were compatible with those of the transfectant Ba/F3 cell lines. Our findings suggest that NSCLC harboring the EGFR L861Q mutation might be sensitive to afatinib or osimertinib and that NSCLC harboring the EGFR S768I mutation might be sensitive to afatinib. Overall, afatinib might be the optimal EGFR-TKI against these uncommon EGFR mutations.

Activating mutations in the epidermal growth factor receptor (EGFR) gene occur in 40% of non-small cell lung cancer (NSCLC) patients among Asians1 and in 20% of those among Caucasians.2,3 The most common EGFR mutations are in-frame exon 19 deletions and the exon 21 L858R point mutation, which constitute approximately 90% of all EGFR mutations.4 Most patients with NSCLC harboring exon 19 deletions or the L858R mutation respond dramatically to the first generation (1G) reversible EGFR tyrosine kinase inhibitors (EGFR-TKI) gefitinib and erlotinib5–9 and to the second generation (2G) irreversible EGFR-TKI afatinib.10,11 Recently, third generation (3G) EGFR-TKI, which are mutant-selective and irreversible inhibitors, have been developed.12–16 3G EGFR-TKI are reportedly effective for patients with NSCLC harboring the EGFR T790M mutation, which is the most common mechanism of acquired resistance to EGFR-TKI.13–16 To date, both the experimental and clinical efficacy of various types of EGFR-TKI for common EGFR mutations have been reported, while less information about the sensitivities of uncommon EGFR mutations is available. The uncommon EGFR mutations, which include exon 18 mutations, S768I in exon 20, L861Q in exon 21 or insertions in exon 20, account for approximately 10% of EGFR mutations in NSCLC.17 Several studies, although very small, have shown that 1G EGFR-TKI are less effective in patients with NSCLC harboring such uncommon EGFR mutations, compared with patients harboring common EGFR mutations.18–26 In contrast, our previous study, in which the sensitivities to various EGFR-TKI for exon 18 mutations were investigated in vitro, showed the efficacy of afatinib or neratinib against exon 18 mutations.27 Indeed, afatinib was reported to be clinically effective for patients with NSCLC harboring uncommon EGFR mutations, including exon 18 mutations in a recent post-hoc analysis.28 Although this study also indicated the efficacy of afatinib in patients with NSCLC harboring EGFR L861Q or S768I mutations, it remains unclear what EGFR-TKI is
optimal for patients with NSCLC harboring these uncommon EGFR mutations. In this study, we focused on the EGFR L861Q and S768I mutations and investigated the in vitro sensitivities of cells carrying these mutations to various EGFR-TKI.

Materials and Methods

Structure of epidermal growth factor receptor. The crystal structure of EGFR in this study was modified based on the crystal structure (ID, 4G5J) deposited in the Protein Data Bank (PDB), which showed the structure of wild-type EGFR in complex with afatinib. The modified picture was drawn using the PyMOL Molecular Graphics System (Version 1.7.4) (Schrodinger, New York, NY, USA), as previously described.

Cell culture and reagents. The Ba/F3 cell line was maintained in IL-3 additive RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS (Sigma-Aldrich). Conditioned medium from WEHI-3 cells was used as a source of IL-3, as previously described. The KYSE270 and KYSE450 cell lines (human esophageal cancer cell lines) were maintained in a 1:1 mixture of RPMI1640 and F12 (Nissui Pharmaceutical, Tokyo, Japan) with 2% FBS according to a previously reported method.

According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), these cell lines carry uncommon EGFR mutations (KYSE270, L861Q and KYSE450, S768I, respectively). All the cell lines were cultured in a 5% CO2-humidified atmosphere at 37°C. Erlotinib, afatinib and osimertinib were purchased from Selleck Chemicals (Houston, TX, USA).

Plasmid construction, viral production and stable transfectants. We constructed retrovirus vectors expressing enhanced green fluorescent protein (EGFP) and EGFR L858R, L861Q and S768I. The methods used in this section have been previously described. The retroviral vector pBABE, carrying the full-length cDNA of wild-type EGFR, was purchased from Addgene (Cambridge, MA, USA). A pBABE construct encoding EGFR L858R, L861Q or S768I was then generated using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Shiga, Japan). The primers used for introducing each mutant EGFR were as follows: L858R-F, GCCGGCCCAAACCTGCTGGTGC; L858R-R, AGCAGTTTGCCCGCCCAAATCCTGATTGTG; L858R-1, GCCCGGGAAGAAGAAGAAGA; L861Q-1, ACCCAGGCTCCAGCAAACTTCAATGC; L861Q-2, GTGCCACATCAGTGCCTCACCT; S768I-1, ATGCCATCGTGCACGCGCAG; S768I-2, ATGCCATCGTGCACGCGCAG.

All the mutations were confirmed by sequencing. The stable viral transfectant Ba/F3 cell lines were designated as Ba/F3-EGFP, Ba/F3-L858R, Ba/F3-L861Q and Ba/F3-S768I, respectively.

Western blot analysis. A western blot analysis was performed as described previously. Rabbit antibodies specific for EGFR, phospho-EGFR, caspase-3, cleaved-caspase-3 and β-actin were obtained from Cell Signaling (Beverly, MA, USA). To evaluate the influence of reagents on phosphorylation and apoptosis, the cells were maintained in a 5% CO2-humidified atmosphere at 37°C. The cell growth was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay, as described previously.

In vitro growth inhibition assay. The growth-inhibitory effects of various EGFR-TKI were examined using an MTT assay, as described previously. When the transfectant Ba/F3 cell lines were used, the cells were cultured without IL-3. The experiment was performed in triplicate.

Statistical analysis. The results of experiments were presented as the means ± SD or the mean of independent triplicate experiments and were analyzed using the Student t-test. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA, USA). A P-value less than 0.05 was considered statistically significant.

Results

EGFR L861Q or S768I mutations have an oncogenic activity similar to that of the EGFR L858R mutation. The crystal structure of EGFR was drawn using the PyMOL Molecular Graphics System based on crystal structure information from PDB ID 4G5J. The structure of EGFR is shown in Figure 1a. Codon 768 is located in the alpha-C helix, and both codons 858 and 861 are located in the activation loop. To assess the effects of EGFR-TKI on these EGFR mutations properly, Ba/F3 cell lines harboring each EGFR mutation (L858R, L861Q, or S768I) were created using a retroviral method. EGFR overexpression was confirmed by a western blot analysis (Fig. 1b), and an IL-3 independent cell growth assay was performed. The Ba/F3 cell line is well known to be dependent on IL-3 but to be rendered IL-3 independent by induction with activating mutations of tyrosine kinase oncogenes. Although the Ba/F3 and Ba/F3-EGFP cell lines could not grow in the absence of IL-3, all the Ba/F3 cell lines harboring each EGFR mutation (L858R, L861Q or S768I) were able to grow IL-3 independently (Fig. 1c). These results indicate that both EGFR L861Q and S768I are activating oncogenic mutations similar to the common mutation EGFR L858R.

Different sensitivities to various epidermal growth factor receptor-tyrosine kinase inhibitors in each transfectant Ba/F3 cell line. The Ba/F3 cell line is often used as a model system for assessing the effects of kinase oncogenes and their sensitivities to inhibitors. Therefore, to investigate the sensitivities to various EGFR-TKI, growth inhibition assays in each transfectant Ba/F3 cell line were performed using an MTT assay. To evaluate the difference in sensitivities to various EGFR-TKI, erlotinib, afatinib and osimertinib were used, because these EGFR-TKI are widely used in clinical settings. The 50% inhibitory concentration (IC50) values of all the EGFR-TKI examined in this study are summarized in Table 1. The Ba/F3-L861Q and Ba/F3-S768I cell lines were less sensitive to erlotinib, compared with the Ba/F3-L858R cell line. In contrast, their sensitivities to afatinib were similar to that of the Ba/F3-L858R cell line. The Ba/F3-L861Q cell line was similarly sensitive and the Ba/F3-S768I cell line was less sensitive to osimertinib, compared with the Ba/F3-L858R cell line (Fig. 2a).

Next, we estimated the IC50 ratios relative to the Ba/F3-L858R cell line to evaluate the difference in drug sensitivities to various EGFR-TKI in each EGFR mutation distinctly. As shown in Figure 2b, the IC50 values of erlotinib in both the Ba/F3-L861Q and Ba/F3-S768I cell lines were much greater than that of the Ba/F3-L858R cell line (IC50 ratio, 20-fold and 32-fold, respectively). In contrast, there was no such difference in the IC50 values of afatinib among the Ba/F3-L858R, Ba/F3-L861Q and Ba/F3-S768I cell lines. The IC50 ratios of afatinib in the Ba/F3-L861Q and Ba/F3-S768I cell lines relative to the
Ba/F3-L858R cell line were less than 5-fold (2.5-fold and 3.5-fold, respectively) (Fig. 2b). The IC_{50} value of osimertinib in the Ba/F3-L861Q cell line was not relatively high, compared with that of the Ba/F3-L858R cell line (IC_{50} ratio, 3.6-fold), whereas the IC_{50} value of osimertinib in the Ba/F3-S768I cell line was much higher than that in the Ba/F3-L858R cell line (IC_{50} ratio, 20-fold) (Fig. 2b). These results suggest that NSCLC harboring the EGFR L861Q mutation might be sensitive to afatinib or osimertinib, and that NSCLC harboring the EGFR S768I mutation might be sensitive to afatinib.

**Differences in epidermal growth factor receptor inhibitory effects of various epidermal growth factor receptor-tyrosine kinase inhibitors among EGFR L858R, L861Q, and S768I mutations.** Next, to estimate the EGFR inhibitory effects of these EGFR-TKI for each EGFR mutation, western blot analyses were performed. Afatinib inhibited the phosphorylation of EGFR to almost the same degree in all of the transfectant Ba/F3 cell lines, whereas erlotinib inhibited the phosphorylation of EGFR to a lesser degree in both the Ba/F3-L861Q and Ba/F3-S768I cell lines than in the Ba/F3-L858R cell line (Fig. 3). In particular, the phosphorylation of EGFR persisted in the Ba/F3-L861Q and Ba/F3-S768I cell lines even in the presence of 100 nM of erlotinib (Fig. 3). Osimertinib inhibited the phosphorylation of EGFR to almost the same degree in the Ba/F3-L861Q cell line but to a lesser degree in the Ba/F3-S768I cell line, compared with that in the Ba/F3-L858R cell line (Fig. 3). The phosphorylation of EGFR also strongly persisted in the Ba/F3-S768I cell line in the presence of 100 nM of osimertinib (Fig. 3). These results are consistent with those of the growth inhibitory assays, indicating that the difference in sensitivities to various EGFR-TKI is caused by the difference in the EGFR inhibitory effects of various EGFR-TKI on each EGFR mutation.

**KYSE270 and KYSE450 cell lines exhibit a tendency similar to that of the transfectant Ba/F3 cell lines.** Next, we investigated the effects of various EGFR-TKI in EGFR-mutated cancer cell lines to confirm the experimental results that were obtained using the Ba/F3 cell lines. No NSCLC cell line harboring uncommon EGFR mutations, such as EGFR L861Q or S768I mutations, could be found. Instead, the esophageal cancer cell lines KYSE270 (L861Q) and KYSE450 (S768I) were used to evaluate the sensitivities to various EGFR-TKI, the EGFR signal and apoptosis. Both the KYSE270 and KYSE450 cell lines were most sensitive to afatinib and least sensitive to erlotinib (Fig. 4a). Both cell lines were intermediate-sensitive to osimertinib. Among them, osimertinib was as effective against the KYSE270 cell line as afatinib but was much less effective than afatinib against the KYSE450 cell line (Fig. 4a). The IC_{50} values of erlotinib, afatinib and osimertinib in the KYSE270 cell line were 0.7, 0.004 and 0.03 μM, respectively, and those of erlotinib, afatinib and osimertinib in the KYSE450 cell line were 1.1, 0.004 and 0.4 μM, respectively (Fig. 4b).

In both cell lines, afatinib markedly inhibited the phosphorylation of EGFR, compared with erlotinib (Fig. 5a). Osimertinib immediately inhibited the phosphorylation. In particular, in the KYSE450 cell line, its EGFR inhibitory effect was much weaker than that of afatinib (Fig. 5a). In the KYSE270 cell

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**Table 1. IC_{50} values of various EGFR-TKIs in the transfectant Ba/F3 cell lines**

<table>
<thead>
<tr>
<th>EGFR-TKI (nM)</th>
<th>EGFR mutation</th>
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<tbody>
<tr>
<td></td>
<td>L858R</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>4.5</td>
</tr>
<tr>
<td>Afatinib</td>
<td>0.2</td>
</tr>
<tr>
<td>Osimertinib</td>
<td>2.5</td>
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IC_{50}, 50% inhibitory concentration; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.
line, afatinib and osimertinib elevated the expression level of cleaved caspase-3, whereas only afatinib elevated this level in the KYSE450 cell line (Fig. 5b). Based on the results of western blot analyses, the difference in the EGFR inhibitory effects and the induction of apoptosis reflected the different sensitivities to various EGFR-TKI. These results were compatible with those of the transfectant Ba/F3 cell lines.

Discussion

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for 20% of all cancer-related deaths.\(^{(37,38)}\) NSCLC accounts for approximately 85% of all lung cancers, and the EGFR mutation frequency in patients with NSCLC is relatively high (Asia, 47%; North America, 22%; and Europe, 15%).\(^{(38)}\) Therefore, NSCLC harboring uncommon EGFR mutations should not be overlooked, although uncommon EGFR mutations account for only 10% of all EGFR mutations in NSCLC.\(^{(17)}\) In the present study, the uncommon EGFR mutations L861Q and S768I were focused on, and the in vitro differences in sensitivities to various EGFR-TKI and the efficacy of afatinib for both EGFR L861Q and S768I mutations were revealed for the first time.

Most studies examining uncommon EGFR mutations are small retrospective studies or case reports, and clinical data is limited because this population is “uncommon” (G719X, 5%; S768I, 1%; L861Q, 3%; exon 20 insertions, 3%).\(^{(17)}\) Although several articles have reported patients with NSCLC harboring uncommon mutation who responded to 1G EGFR-TKI, getitinib or erlotinib,\(^{(39,40)}\) many studies have shown that these uncommon EGFR mutations were less sensitive to 1G EGFR-TKI (objective response rate, less than 50%), compared with common EGFR mutations.\(^{(18-26)}\) Preclinical studies showing a lower efficacy of 1G EGFR-TKI for such uncommon mutations supports these clinical data.\(^{(27,41-43)}\) Our previous study has shown that EGFR exon 18 mutations are less sensitive to 1G EGFR-TKI, compared with common mutations,\(^{(27)}\) and a similar tendency for EGFR L861Q or S768I mutations was observed in the present study. These findings are consistent with previous clinical and in vitro studies.\(^{(18-27,37-39)}\) In contrast, a combined post-hoc analysis revealed a favorable progression-free survival and treatment response for afatinib in patients with NSCLC harboring...
uncommon EGFR mutations, including G719X, L861Q and S768I mutations (objective response rate, 77.1%; disease control rate, 84.2%; progression free survival, 10.7 months). (28) Our previous in vitro study demonstrated the efficacy of afatinib or neratinib for the exon 18 mutation, including G719X. (27) Furthermore, in our present study, the EGFR L861Q or S768I mutation was considered to be sensitive to afatinib. Thus, these results were consistent with the favorable outcome of afatinib-treated patients with these uncommon EGFR mutations. (28) In contrast, like 1G EGFR-TKI, afatinib might be less effective for most exon 20 insertions in preclinical and clinical studies. (24,25,28,42,43) Few reports regarding the effectiveness of 3G EGFR-TKI against patients with NSCLC harboring uncommon EGFR mutations are available. Recent preclinical studies revealed that osimertinib might be partially effective for exon 20 insertions. (42,43) Our previous in vitro study showed a lower efficacy of 3G EGFR-TKI, including osimertinib, for EGFR exon 18 mutations, (27) and the present in vitro study indicated that osimertinib might be effective against the EGFR L861Q mutation, but is likely to be less effective against the EGFR S768I mutation. Considering these findings including our studies, (18–23,27,28,41) afatinib might be optimal for patients with NSCLC harboring the EGFR uncommon mutations including exon 18 mutations, L861Q and S768I in clinical settings.

We also found that the different EGFR inhibitory effects of EGFR-TKI reflected the different sensitivities to various EGFR-TKI. Several studies, in which affinities between kinases and various EGFR-TKI were investigated, indicate that the different EGFR inhibitory effects of EGFR-TKI might be associated with the different structural-based interactions. (25,44) However, a similar study for 3G EGFR-TKI has not yet been reported. Both 2G and 3G EGFR-TKI are irreversible small-molecule inhibitors that covalently bind to EGFR kinase by targeting the cysteine-797 residue, located in the ATP binding pocket, and 3G EGFR-TKI are considered to be more specific for EGFR sensitizing mutations than 2G EGFR-TKI. (12–14) Nevertheless, in our studies, 3G EGFR-TKI seemed to be less effective against the uncommon EGFR mutations. Although the detailed mechanism remains unclear, we speculated that this difference in the response of cell lines harboring uncommon EGFR mutations to afatinib and osimertinib might be caused by different structural-based interactions based on the present findings. Further investigations, including structural analyses, are needed to clarify our hypothesis.

This study had several limitations. First, neither the binding affinities between tyrosine kinases and EGFR-TKI nor the protein structures could be analyzed. Instead, we determined the

**Fig. 3.** Difference in epidermal growth factor receptor (EGFR) inhibitory effects of various EGFR-TKI among the transfectant Ba/F3 cell lines (L858R, L861Q and S768I). Three hours after the cells were treated with the indicated concentrations of drugs, the samples were collected. Afatinib inhibited the phosphorylation of EGFR to almost the same degree in all the transfectant Ba/F3 cell lines, whereas erlotinib inhibited the phosphorylation of EGFR to a lesser degree in both the Ba/F3-L861Q and Ba/F3-S768I cell lines, compared with the Ba/F3-L858R cell line. Osimertinib inhibited the phosphorylation of EGFR to almost the same degree in the Ba/F3-L861Q cell line and to a lesser degree in the Ba/F3-S768I cell line, compared with that in the Ba/F3-L858R cell line. β-actin was used as an internal control. p-EGFR, phospho-EGFR.

**Fig. 4.** Sensitivities to various EGFR-TKI in the KYSE270 and KYSE450 cell lines. (a) Growth inhibitory curves of various EGFR-TKI in the KYSE270 and KYSE450 cell lines. The cells were exposed to each concentration of various EGFR-TKI for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. Both the KYSE270 (L861Q) and KYSE450 (S768I) cell lines were most sensitive to afatinib and least sensitive to erlotinib. Osimertinib was as effective for the KYSE270 cell line as afatinib, but was much less effective for the KYSE450 cell line than afatinib. Lines, mean of independent triplicate experiments. (b) IC50 values of various EGFR-TKI in the KYSE270 and KYSE450 cell lines. The IC50 values of erlotinib, afatinib and osimertinib in the KYSE 270 cell line (L861Q) were 0.7, 0.004 and 0.03 μM, respectively, while those of erlotinib, afatinib and osimertinib in the KYSE 450 cell line (S768I) were 1.1, 0.004 and 0.4 μM, respectively.
crystal structure of EGFR using the PDB database. Second, we did not use NSCLC cell lines, but rather artificially transfected Ba/F3 cell lines and esophageal cancer cell lines, because we could not find NSCLC cell lines harboring uncommon EGFR mutations such as the EGFR L861Q or S768I mutations. However, the Ba/F3 cell line has often been used in many studies investigating EGFR mutations, and trends similar to the results obtained using transfectant Ba/F3 cell lines were confirmed in the KYSE cell lines, even though they are not NSCLC cell lines. Third, we could not analyze clinical data or samples. The number of patients with NSCLC harboring such uncommon mutations is too small to show such data. Large-scale clinical trials should be performed but might be difficult in such small patient subgroups. Therefore, studies, like ours, investigating the in vitro sensitivities to various EGFR-TKI might be valuable.

In conclusion, based on our present in vitro findings, NSCLC harboring the EGFR L861Q mutation might be sensitive to afatinib or osimertinib, and NSCLC harboring the EGFR S768I mutation might be sensitive to afatinib. Our previous study also indicated that afatinib or neratinib might be effective against NSCLC harboring EGFR exon 18 mutations. Taken together, these findings suggest that afatinib might be optimal for NSCLC patients harboring the uncommon EGFR mutations including exon 18 mutations, L861Q and S768I in clinical settings. Our in vitro study may help clinicians to select an appropriate EGFR-TKI in such subsets of NSCLC patients. To confirm these findings, further clinical and basic research is needed.

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