Afatinib is Especially Effective Against Non-small Cell Lung Cancer Carrying an *EGFR* Exon 19 Deletion

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Abstract. *Background: A recent pooled analysis of the LUX-LUNG3 and LUX-LUNG6 trials suggested that afatinib (an irreversible epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI)) is especially effective against non-small cell lung cancer (NSCLC) carrying an EGFR exon 19 deletion. Materials and Methods: Stable viral transfectant HEK293 cell lines carrying an exon 19 deletion (HEK293/19 del) or exon 21 L858R mutation (HEK293/ L858R)) were created and their drug sensitivities to AG1478 (a reversible EGFR-TKI) and afatinib were examined using an MTT assay. Western blot analyses were performed to estimate the phosphorylation of EGFR. Results: In the HEK293/19 del, the 50% inhibitory concentration (IC_{50}) of afatinib was significantly lower than that in the HEK293/ L858R. In addition, afatinib inhibited the phosphorylation of EGFR to a greater degree in the HEK293/19 del than in the HEK293/ L858R. Conclusion: Our experimental findings suggest that afatinib is especially effective against NSCLC carrying an EGFR exon 19 deletion.*

Gefitinib and erlotinib are first-generation reversible epidermal growth factor receptor (*EGFR*) -tyrosine kinase inhibitors (*EGFR*-TKIs) that are highly effective against non-small cell lung cancer (NSCLC) carrying activating *EGFR* mutations (1-3). In addition, afatinib, a second-generation irreversible EGFR-TKI, also exhibits a marked efficiency against NSCLC carrying *EGFR* mutations, similar to the effect of gefitinib and erlotinib (4, 5). In the LUX-LUNG3 and LUX-LUNG6 trials, in which afatinib was compared with platinum-doublet chemotherapy as a first-line therapy, the progression-free survival (PFS) was significantly longer in the afatinib group than in the platinum-doublet group (4, 5). Furthermore, a recent pooled analysis of the LUX-LUNG3 and LUX-LUNG6 trials showed a longer overall survival (OS) in the afatinib group than in the platinum-doublet group, although gefitinib or erlotinib (but not afatinib) was used for approximately 60% of the platinum-doublet group as a second-line or later therapy (6). In a sub-group analysis, the improvement in the OS was especially notable among patients with NSCLC carrying an *EGFR* exon 19 deletion but not in patients with NSCLC carrying the *EGFR* exon 21 L858R mutation. These findings suggested that afatinib is more effective than gefitinib or erlotinib against NSCLC carrying the *EGFR* exon 19 deletion. However, the supportive evidence remains insufficient. In the present study, we investigated the supportive evidence using *EGFR*-mutated NSCLC cell lines and stable viral transfectant HEK293 cell lines with equal *EGFR* expression levels (exon 19 deletion or exon 21 L858R).

Materials and Methods

Cell culture and reagents. The PC-9, HCC827 (*EGFR* exon 19 deletion) and 11_18 (*EGFR* exon 21 L858R) cell lines (human NSCLC cell lines; National Cancer Center, Tokyo, Japan) were maintained in RPMI1640 medium with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). The HEK293 cell line (human embryonic kidney cell line) was maintained in DMEM medium (Nissui Pharmaceutical, Tokyo, Japan) with 10% FBS. All the cell lines were maintained in a 5% CO2-humidified atmosphere at 37˚C. AG1478 (a reversible *EGFR*-TKI) and BIBW2992 (afatinib) were purchased from Selleck (Houston, TX, USA).

In vitro growth inhibition assay. The growth-inhibitory effect of AG1478 and of BIBW2992 was examined using an MTT (Sigma-Aldrich) assay as described previously (7).

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Table I. *IC_{50} values of AG1478 and BIBW2992 (afatinib) in each cell line.*

Cell lines	*EGFR* status	IC_{50}		
		AG1478	BIBW2992	Ratio
PC-9	Exon 19 deletion	17 nM	0.28 nM	60.7
HCC827	Exon 19 deletion	6.6 nM	0.31 nM	21.3
11_18	Exon 21 L858R	0.30 μM	0.085μM	3.53
HEK293/19 del	Exon 19 deletion	19.5 μM	0.011 μM	1772.7
HEK293/L858R	Exon 21 L858R	21.3 μM	0.64 μM	33.3

EGFR, Epidermal growth factor receptor gene; IC_{50}, 50% inhibitory concentration; Ratio, IC_{50} of AG1478 relative to that of BIBW2992.

Plasmid construction, viral production and stable transfectants. The methods used in this section have been previously described (8). Briefly, a full-length cDNA fragment was introduced into a pQCLIN retroviral vector (Clontech; Palo Alto, CA, USA) together with enhanced green fluorescent protein (EGFP) following the internal ribosome entry site sequence (IRES) to monitor the expression of the inserts indirectly. The vectors and the stable viral transfectant HEK293 cell lines were designated as pQCLIN-EGFP, pQLCIN-*EGFR* wild-type, pQLCIN-*EGFR* exon 19 deletion, pQLCIN-*EGFR* exon 21 L858R, HEK293/EGFP, HEK293/WT, HEK293/19 del and HEK293/L858R, respectively.

Western blot analysis. A Western blot analysis was performed as described previously (7). Rabbit antibodies specific for *EGFR*, phospho-*EGFR*, and β-actin were obtained from Cell Signaling (Beverly, MA, USA). To evaluate the influence of reagents on the phosphorylation, the cells were stimulated for 3 hours.

Results

We first examined the sensitivities to AG1478 (a reversible *EGFR*-TKI) and BIBW2992 (afatinib) in NSCLC cell lines carrying the *EGFR* exon 19 deletion (PC-9 and HCC827 cell lines) or the exon 21 L858R mutation (11_18 cell line) using an MTT assay. In all the cell lines, the 50% inhibitory concentrations (IC_{50}) of BIBW2992 were lower than those of AG1478. The IC_{50} ratios (IC_{50} of AG1478/BIBW2992) of the PC-9 and HCC827 cell lines were both more than 20, whereas that of the 11_18 cell line was less than 5. These findings suggest that afatinib is especially effective against NSCLC cell lines carrying the *EGFR* exon 19 deletion (Table I).

Next, *EGFR* (wild-type, exon 19 deletion or exon 21 L858R mutation)-overexpressed HEK293 cell lines were created. Clones with equivalent levels of EGFP-positivity were selected to establish cell lines with equivalent levels of *EGFR* expression (Figure 1A). In the HEK293/19 del cell line, the IC_{50} of AG1478 was equivalent to that in the HEK 293/L858R cell line but the IC50 of BIBW2992 was lower than that in the HEK293/L858R cell line (Table I).

Furthermore, using a Western blot analysis, BIBW2992 inhibited the phosphorylation of *EGFR* to a greater degree in the HEK293/19 del cell line than in the HEK293/L858R cell line, whereas AG1478 inhibited the phosphorylation of *EGFR* to the same degree in both cell lines (Figure 1B). These findings suggest that NSCLC carrying the *EGFR* exon 19 deletion is more sensitive to afatinib since this reagent strongly inhibited the phosphorylation of *EGFR* carrying the exon 19 deletion.

Discussion

The most common activating *EGFR* mutations in patients with NSCLC include short in-frame deletions in exon 19 (exon 19 deletion) and a specific point mutation in exon 21 at codon 858 (exon 21 L858R). Both mutations account for approximately 80%–90% of all the *EGFR* mutations that have been detected (9). In several clinical trials in which gefitinib or erlotinib was compared with platinum-doublet chemotherapy as a first-line therapy in patients with NSCLC and *EGFR* mutations, the OS did not differ significantly between the gefitinib or erlotinib group and the platinum-doublet therapy group, despite a significant difference in the PFS (10-14). These results seemed to be caused by more than half of the patients crossing over to the alternative therapy as a second-line or later therapy. In contrast, a recent pooled analysis of the LUX-LUNG3 and LUX-LUNG6 trials showed a significantly longer OS in the afatinib group than in the platinum-doublet group, although gefitinib or erlotinib (but not afatinib) was used in approximately 60% of the platinum-doublet group as a second-line or later therapy (4-6). This analysis also showed that the improvement in OS was particularly notable in patients with NSCLC carrying an *EGFR* exon 19 deletion (hazard ratio (HR)=0.59; 95% confidence interval (CI)=0.45-0.77; $p<0.001$) but no improvement was observed in patients with NSCLC carrying the *EGFR* exon 21 mutation (HR=1.25; 95%CI=0.92-1.71; P=0.160) (6). These results suggested that afatinib has a greater anticancer activity than gefitinib or erlotinib in patients with NSCLC carrying an *EGFR* exon 19 deletion.

Our present study showed that the IC_{50} of afatinib was lower than that of AG1478 (a reversible EGFR-TKI) in all the cell lines with *EGFR* mutations, including the HEK293 cell lines, but the cell lines carrying an *EGFR* exon 19 deletion were highly sensitive to afatinib. In addition, afatinib inhibited the phosphorylation of *EGFR* to a greater degree in the HEK293/19 del cell line than in the HEK293/L858R cell line, whereas AG1478 inhibited the phosphorylation of *EGFR* to the same degree in both the exon19 deletion and L858R cell lines. These findings support the hypothesis that afatinib is more effective against NSCLC carrying the *EGFR* exon 19 deletion. Several studies describing differences between the *EGFR* exon 19 deletion and the exon 21 L858R mutation have also been published (15). Therefore, we speculated that such differences between these mutations might be associated

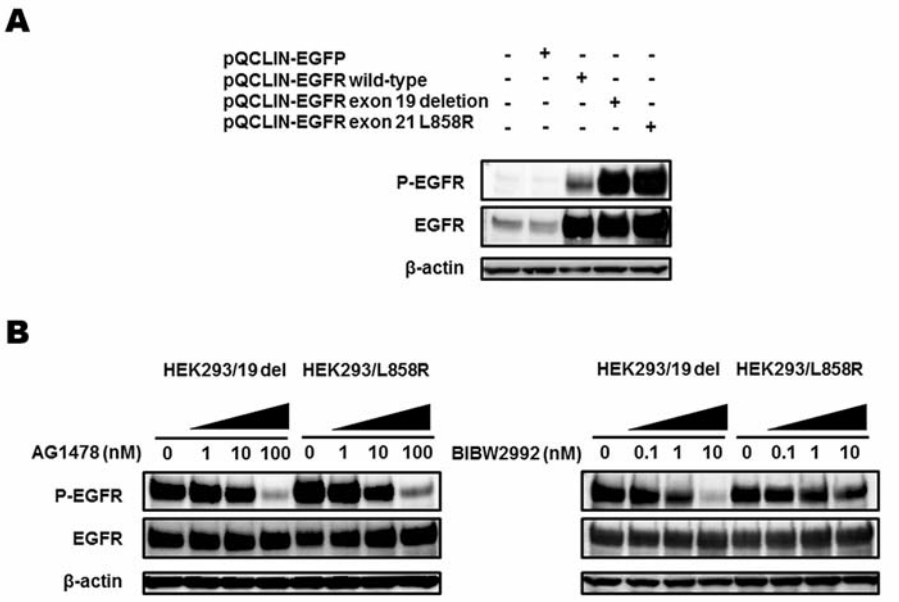

Figure 1. *Influence of AG1478 or BIBW2992 (afatibib) on the phosphorylation in EGFR (exon 19 deletion, and exon 21 L858R)-overexpressed HEK293 cell lines. A, Western blot analyses in the HEK293 transfectant cell lines. The expression of EGFR was almost equivalent in each cell line and EGFR of HEK293/19 del and HEK293/L858R was more phosphorylated than that of HEK293/WT. β-actin was used as an internal control. B, Western blot analyses in the HEK293/19 del and HEK293/L858R cell lines. The cells were treated with AG1478 or BIBW2992 for 3 hours before sample collection. BIBW2992 inhibited the phosphorylation of EGFR to a greater degree in the HEK293/19 del cell line than in the HEK293/L858R cell line, whereas AG1478 inhibited the phosphorylation of EGFR to the same degree in both cell lines. β-actin was used as an internal control.*

with our findings; however, the detailed mechanism remains unclear and further research is needed.

In conclusion, our findings indicated that afatinib might have a greater anticancer activity against NSCLC carrying an *EGFR* exon 19 deletion since this reagent strongly inhibits the phosphorylation of *EGFR* carrying the exon 19 deletion. These findings support the improvement in the OS of patients with NSCLC bearing the *EGFR* exon 19 deletion who receive afatinib treatment, as has been reported in a recent pooled analysis of the LUX-LUNG3 and LUX-LUNG6 trials. To confirm these findings and to elucidate the detailed mechanism, large prospective studies and further research will be required.

Conflicts of Interest

None declared for this study.

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