Antitumor effect of afatinib, as a human epidermal growth factor receptor 2-targeted therapy, in lung cancers harboring HER2 oncogene alterations

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Human epidermal growth factor receptor 2 (HER2) is a member of the HER family of proteins containing four receptor tyrosine kinases. It plays an important role in the pathogenesis of certain human cancers. In non-small-cell lung cancer (NSCLC), HER2 amplification or mutations have been reported. However, little is known about the benefit of HER2-targeted therapy for NSCLCs harboring HER2 alterations. In this study, we investigated the antitumor effect of afatinib, an irreversible epidermal growth factor receptor (EGFR)–HER2 dual inhibitor, in lung cancers harboring HER2 oncogene alterations, including novel HER2 mutations in the transmembrane domain, which we recently identified. Normal bronchial epithelial cells, BEAS-2B, ectopically overexpressing wild-type HER2 or mutants (A775insYVMA, G776VC, G776LC, P780insGSP, V659E, and G660D) showed constitutive autophosphorylation of HER2 and activation of downstream signaling. They were sensitive to afatinib, but insensitive to gefitinib. Furthermore, we examined the antitumor activity of afatinib and gefitinib in several NSCLC cell lines, and investigated the association between their genetic alterations and sensitivity to afatinib treatment. In HER2-altered NSCLC cells (H2170, Calu-3, and H1781), afatinib downregulated the phosphorylation of HER2 and EGFR as well as their downstream signaling, and induced an antiproliferative effect through G1 arrest and apoptotic cell death. In contrast, HER2- or EGFR-non-dependent NSCLC cells were insensitive to afatinib. In addition, these effects were confirmed in vivo by using a xenograft mouse model of HER2-altered lung cancer cells. Our results suggest that afatinib is a therapeutic option as a HER2-targeted therapy for NSCLC harboring HER2 amplification or mutations.

Lung cancer remains the leading cause of cancer-related death worldwide.1) Recent developments in the genomic characterization of tumors have contributed to novel therapeutic approaches, and some molecular-targeted therapies based on the genetic profiles of tumors have improved patient survival. For example, EGFR TKIs, such as gefitinib or erlotinib, have shown marked antitumor activity in NSCLC patients with an active EGFR mutations, and crizotinib, an ALK TKI, has shown similar promise for NSCLC patients with ALK gene rearrangements.2,3) Furthermore, other oncogenic alterations, such as KRAAS, NRAS, BRAF, MET, and FGFR, have been identified in some subsets of NSCLC.4,5) Human epidermal growth factor 2 (also known as ERBB2) is a member of the HER family containing four receptor tyrosine kinases. This receptor is frequently overexpressed in various human cancers, and many preclinical studies have shown that overexpression of HER2 or mutations of the HER2 kinase domain play an important role in oncogenic transformation and tumorigenesis.6–9) In NSCLC patients, HER2 overexpression and HER2 amplification were reported in 11–32% and 2–23%, respectively.10–13) HER2 mutations were identified in approximately 2–4% of NSCLCs and were usually mutually exclusive with other driver mutations.14,15) Shigematsu et al.15) reported that all HER2 mutations in the tyrosine kinase domain were located in the same loop of exon 20, consisting of A775insYVMA (55%), G776VC (18%), P780insGSP (18%), and G776LC (9%). Recently, we have identified two novel mutations in the transmembrane domain of HER2 encoded by exon 17 (V659E and G660D) in lung adenocarcinomas. Our preliminary data suggested that these mutations might be oncogenic alterations of lung adenocarcinoma.16) However, little is known about the benefit of HER2-targeted therapy for NSCLCs harboring HER2 alterations.

Afatinib, also known as BIBW2992, is an irreversible inhibitor of EGFR and HER2, and has recently been approved for the treatment of EGFR-mutant NSCLCs in several countries. In two randomized phase III trials, afatinib showed significant benefit in progression-free survival compared with standard doublet chemotherapy in NSCLC patients with EGFR mutations.17,18)
In this study, we investigated the efficacy of afatinib as a HER2-targeted therapy in vitro in NSCLC cells harboring HER2 gene amplification or mutations, including novel mutations in the transmembrane domain, and confirmed these antitumor effects in vivo using a xenograft mouse model of HER2-altered lung cancer cells.

Materials and Methods

Cell lines and reagents. Eight lung cancer cell lines (A549, Calu-3, HCC827, NCI-H1299, NCI-H1781, NCI-H1975, NCI-H1993, and NCI-H2170), two breast cancer cell lines (SK-BR-3 and BT-474), and one normal human bronchial epithelial cell line (BEAS-2B) were used in this study. These cell lines, except A549, SK-BR-3, BT-474 and BEAS-2B, were kindly provided by Dr. Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA), who established these lines with Dr. John D. Minna.(19,20) A549, SK-BR-3, and BT-474 were purchased from ATCC (Manassas, VA, USA). BEAS-2B was purchased from European Collection of Cell Cultures (Porton Down, UK). All lung cancer cells were cultured in RPMI-1640 media supplemented with 10% FBS and the other cells were maintained in DMEM with 10% FBS. They were grown in a humidified incubator with 5% CO2 at 37°C. Afatinib and gefitinib were purchased from Sykkinase (San Diego, CA, USA) and InvivoGen (San Diego, CA, USA), respectively.

Plasmid constructs and transfection. Human cDNAs encoding full-length HER2 (wild-type and its variants A775insYVMA, G776VC, G776LC, P780insGSP, V659E, and G660D) were inserted into the pLDT-SMART (C-TSC) vector, pCMViR(Asp214) (Cell Signaling Technology, Danvers, MA, USA), and p44/p42 MAPK, p-p44/p42 MAPK, cleaved PARP (Asp214) (Cell Signaling Technology, Danvers, MA, USA), and β-actin (used as loading control) (Merk Millipore, Billerica, MA, USA). The secondary antibody was HRP-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA). To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-3000 (Fujiﬁlm, Tokyo, Japan).

Western blot analysis. The total cell lysate was extracted with lysis buffer, a mixture of RIPA buffer, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO, USA), and Complete Mini (Roche, Basel, Switzerland). Western blot analysis was carried out by conventional methods using the following primary antibodies: anti-EGFR, phospho-(p)-EGFR (Tyr1068), HER2, p-HER2 (Tyr1221/1222). Akt, p-Akt (Ser473), p44/p42 MAPK, p-p44/p42 MAPK, cleaved PARP (Asp214) (Cell Signaling Technology, Danvers, MA, USA), and β-actin (used as loading control) (Merk Millipore, Billerica, MA, USA). The secondary antibody was HRP-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA). To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-3000 (Fujiﬁlm, Tokyo, Japan).

Copy number analysis by real-time PCR. Copy number gains of EGFR and HER2 were measured by real-time quantitative PCR with the SYBR Green method, using Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA). The antiproliferative activities of each drug were shown as IC50, which is the concentration of the drug required to inhibit cell proliferation by 50%.

Cell cycle analysis. The effects of afatinib on the cell cycle distribution were assessed using a propidium iodide staining-based assay with the CycleTEST PLUS DNA Reagent Kit and FACSCaliber (Becton Dickinson, Franklin Lakes, NJ, USA). Untreated cells and cells treated with 0.1 μM afatinib for 24 or 48 h were subjected to cell cycle analysis. Doubleset, cell debris, and fixation artifacts were gated out, and cell cycle analysis was carried out with CellQuest version 3.1 (Becton Dickinson).

Xenograft model. Six-week-old NOD/SCID female mice were purchased from Charles River Laboratories (Yokohama, Japan). All mice were provided with sterilized food and water, and housed in a barrier facility under a 12:12-h light/dark cycle. Each cell line (5 x 106) was suspended with 200 μL RPMI-1640 media with Matrigel Basement Membrane Matrix (Corning, Corning, NY, USA) and s.c. injected into the backs of the mice. The tumor volume was calculated using the empirical formula \( V = \frac{1}{2} \times (\text{shortest diameter})^2 \times (\text{longest diameter}) \). When the tumors exceeded approximately 50 mm3, mice were randomly allocated to two groups that received either vehicle or 20 mg/kg afatinib. Afatinib was prepared in 0.5 w/v (%) methyl cellulose. Vehicles and afatinib were given orally as a suspension by gavage once daily, 6 days per week. Tumor volume was measured three times a week with calipers. After 4 weeks of treatment, the mice were killed and the tumors were harvested and photographed.

Statistical analysis. Statistical analysis was carried out with GraphPad Prism version 6.0.3, J (GraphPad Software, San Diego, CA, USA). Group differences were compared using one-way ANOVA followed by Dunnett’s test or two-way ANOVA for repeated measurements. P < 0.05 was considered statistically significant.

Results

HER2 alteration activates HER2 signaling, which is inhibited by afatinib. To examine the effect of HER2 alterations on signal transduction pathways, normal bronchial epithelial cells (BEAS-2B) were transiently transfected with wild-type or six mutant HER2-containing vectors, consisting of four kinase domain mutations (A775insYVMA, G776VC, G776LC, and P780insGSP) and two transmembrane domain mutations (V659E and G660D), which were reported in NSCLCs.(15,16)
Cells expressing wild-type and all six HER2 mutants showed strong constitutive autophosphorylation of HER2 in the absence of serum, and stronger phosphorylation of EGFR, which is assumed to be one of the dimerization partners of HER2, and AKT compared with the vector control. Furthermore, all cells expressing HER2-mutants showed increased phosphorylation of HER2, EGFR, and AKT compared with those expressing wild-type (Fig. 1a).

Next, we assessed the affinity changes of afatinib to HER2 kinase domain caused by major HER2 mutations in exon 20. We modeled the 3-D structure of the HER2–afatinib complex and marked the location of major HER2 mutations, which were reported in our previous paper (Fig. 1b).15 Mutations of exon 20 were concentrated in the exposed area on the protein surface (A775 to P780) of the αC-β4 loop (M774 to R784) of the HER2 kinase domain, and this area was located on the back side of the ATP-binding pocket, as shown in the modeled complex structure. This structural feature suggests that the major HER2 mutations in exon 20 could not directly inhibit the binding of ATP and afatinib to the HER2 kinase domain.

We then examined the sensitivity of BEAS-2B cells ectopically expressing wild-type or mutant HER2 to the EGFR–TKI (gefitinib) and EGFR/HER2–TKI (afatinib). Forty-eight hours after transfection, cells were treated with or without gefitinib or afatinib for 24 h, and HER2 signal was assessed using Western blotting. Although gefitinib showed little effect on the phosphorylation of HER2 and EGFR, treatment with afatinib inhibited phosphorylation of HER2, EGFR, and AKT (Fig. 1c). These results indicate that afatinib was more effective than gefitinib in inhibiting HER2-driven signals.

Afatinib inhibits growth of both HER2-amplified and HER2-mutant lung cancer cells. We examined the antitumor activity of afatinib and gefitinib in several NSCLC cell lines, and investigated the association between their genetic alterations and sensitivity to afatinib treatment (Table 1, Fig. 2a). The cell lines of this panel were selected on the basis of previously published genotyping reports19,25 and consisted of two HER2-amplified (NCI-H2170 and Calu-3), one HER2-mutant (NCI-H1781), two EGFR-mutant (NCI-H1975 and HCC827), one KRAS-mutant (A549), one NRAS-mutant (NCI-H1299), and one MET-amplified (NCI-H1993) NSCLC cell lines; two HER2-amplified breast cancer cell lines (SK-BR-3 and BT-474) were also analyzed. We determined the copy number gains of EGFR and HER2 using real-time PCR, and sensitivity was assessed, with the IC50 values for each cell line calculated using an MTS assay. Two NSCLC cell lines, H2170 and Calu-3, and two breast cancer cell lines, SK-BR-3 and BT-474, showed high HER2 amplification, with copy number values of 94.6, 110.9, 23.0, and 32.0, respectively. In contrast, copy number values of HER2 in H1781 cells (HER2-mutant cells) or the other cell lines in this panel did not exceed approximately 3. Next, for drug sensitivity, the proliferation of two HER2-amplified lung cancer cell lines, H2170 and Calu-3, was inhibited by afatinib, with IC50 values of 0.140 and 0.086 μM, respectively. Afatinib also showed strong antiproliferative effects on H1781 cells, with an IC50 of 0.039 μM. Regarding the sensitivity to gefitinib among HER2-altered cells, H2170 and H1781 cells, were resistant to gefitinib (IC50 > 3 μM). In contrast, Calu-3 cells were partially sensitive to gefitinib, with an IC50 of 0.471 μM, which was consistent with a previous report.26 Furthermore, HCC827 cells (EGFR-mutant) were sensitive to afatinib with IC50 values of 0.001 μM, and H1975 cells (EGFR-L858R/T790M mutant) were moderately resistant to afatinib with IC50 values of 0.713 μM. HER2- or EGFR-non-dependent NSCLC cells A549 (KRAS-mutant cells), H1299 (NRAS-mutant cells), and H1993 (MET-amplified cells), were insensitive (IC50 > 2 μM). In addition, afatinib showed antiproliferative effects on HER2-amplified breast cancer cell lines (SK-BR-3 and BT-474 cells), with an IC50 of 0.002 μM for each cell line.

Next, we assessed the effect of afatinib on signal transduction pathways in HER2-amplified or HER2-mutant lung cancer cells. After 6 h of treatment with afatinib, cells were lysed followed by Western blot analysis. As shown in Figure 2(b), afatinib potently inhibited phosphorylation of both HER2 and EGFR at a concentration of 0.1 μM, and the downstream signals, phosphorylation of AKT and MAPK, were also inhibited in both HER2-amplified and HER2-mutant lung cancer cells. Taken together, these results suggest that afatinib showed strong antitumor activity in both HER2-amplified and HER2-mutant lung cancer cells in vitro.

Afatinib induces cell cycle arrest and apoptosis in HER2-dependent cells. To assess the mechanism of growth inhibition, we then examined the effect of afatinib on the cell cycle and apoptosis in HER2-amplified (H2170 and Calu-3 cells), HER2-mutant (H1781 cells), and HER2- or EGFR-non-dependent lung cancer cells (A549 cells). Cells were treated with 0.1 μM afatinib for 24 or 48 h, and analyzed by flow cytometry. To analyze cell cycle distribution, the sub-G1 fraction was excluded and the percentages of each phase were measured (Fig. 3a). Treatment with afatinib caused an increase in the number of H2170, Calu-3, and H1781 cells occupying the G1 phase relative to untreated cells, whereas it was not detected in A549 cells. Next, we investigated the ability of afatinib to induce apoptosis. Cell apoptosis was analyzed with cleaved PARP antibody, as an apoptosis marker, by Western blotting. Afatinib (0.1 μM) induced apoptosis in a time-dependent manner in H2170, Calu-3, and H1781 cells; however, A549 cells did not express cleaved PARP with the treatment (Fig. 3b). These results showed that afatinib induced antiproliferative effects through G1 arrest and apoptotic cell death in HER2-altered cells (H2170, Calu-3, and H1781 cells), whereas HER2- or EGFR-non-dependent NSCLC cells were insensitive to afatinib.

Antitumor effect of afatinib on a xenograft mouse model of HER2-altered cells. On the basis of our in vitro data, we investigated the antitumor effect of afatinib in a xenograft mouse model of HER2-driven lung cancer. H2170 cells (HER2-amplified cells) and H1781 cells (HER2-mutant cells) were used in this experiment. The dose of afatinib was chosen on the basis of previous reports.19,27 One week after transplantation, when tumors exceeded approximately 50 mm3, we started to orally administer vehicle alone or afatinib at 20 mg/kg for 6 days per week. As shown in Figure 4, afatinib treatment significantly inhibited the tumor growth of both H2170 and H1781 xenografts compared with the vehicle control (P < 0.0001 by repeated measurements of ANOVA).

Discussion

Human epidermal growth factor receptor 2 is frequently overexpressed in various human cancers, and targeted therapies for HER2-positive tumors have been proven clinically effective in breast and gastric cancers.28,29 However, it is still unclear whether HER2-targeted therapy shows clinical benefit in NSCLC patients with HER2 aberrations. Previous clinical trials failed to show a clinical benefit with trastuzumab monotherapy or an additional effect of trastuzumab to conventional first-line chemotherapy in HER2-positive NSCLC patients by immuno-
Fig. 1. Overexpressing wild-type or mutant HER2 activates human epidermal growth factor receptor 2 (HER2) signaling, and afatinib inhibits them. (a) BEAS-2B cells were transiently transfected with wild-type HER2, its variants, A775insYVMA, G776VC, G776LC, P780insGSP, G660D, and V659E, or vector control. Approximately 36 h after the transfection, cells were serum-starved overnight. Lysates were subjected to Western blot analysis with the indicated antibodies. (b) Location of the major mutation site and the ATP-binding pocket of the HER2 kinase domain in the modeled HER2–afatinib complex structure. Afatinib is shown as a stick. Residues at the mutation site of the HER2 kinase domains (A775, G776, and P780) are shown as spheres. These residues are concentrated in an exposed area on the protein surface (A775–P780) of the αC–β4 loop (M774–R784), which is located on the back side of the ATP-binding pocket. (c) Forty-eight hours after the transfection, cells were treated with or without 1.0 μM gefitinib or 0.1 μM afatinib for 6 h. Lysates were subjected to Western blot analysis with the indicated antibodies. EGFR, epidermal growth factor receptor.
However, a subgroup of patients with high HER2 expression (immunohistochemistry 3+, HER2 gene amplification, or HER2 mutations were observed to have a good response to HER2-targeted treatment. \(^{(30,33,34)}\) Patient selection based on molecular profiles of tumor cells is important for achieving the maximum benefit of the targeted therapy, because preferable responses can be diluted easily without optimal patient selection. Therefore, to determine the optimal patient selection for HER2-driven NSCLCs, further clinical and preclinical investigations are required.

### Table 1. Characteristics and IC\(_{50}\) values for afatinib and gefitinib in non-small-cell lung cancer (NSCLC) and breast cancer cell lines

| Histologic type | Cell line | Histologic subtype | Gene copy number | Mutation status | Genetic alteration | IC\(_{50}\) μM |
|-----------------|-----------|--------------------|------------------|----------------|-------------------|-------------|
|                 |           |                    | EGFR  | HER2  | EGFR  | HER2  |                       | Afatinib | Gefitinib |
| NSCLC           | H2170     | SQ                 | 2.0   | 94.6  | WT    | WT    | HER2 amplification     | 0.140    | 3.114     |
|                 | Calu-3    | AD                 | 4.0   | 110.9 | WT    | WT    | HER2 amplification     | 0.086    | 0.471     |
|                 | H1781     | AD                 | 2.1   | 2.6   | WT    | WT    | HER2 mutation          | 0.039    | 3.565     |
|                 | HCC827    | AD                 | 48.7  | 2.3   | WT    | WT    | EGFR mutation          | 0.001    | 0.045     |
|                 | H1975     | AD                 | 3.9   | 2.5   | WT    | WT    | EGFR mutation          | 0.713    | >10        |
|                 | H1299     | AD                 | 2.8   | 3.1   | WT    | WT    | K-ras mutation         | 5.275    | >10        |
|                 | A549      | AD                 | 2.1   | 1.5   | WT    | WT    | N-ras mutation         | 4.667    | >10        |
|                 | H1993     | AD                 | 2.9   | 1.8   | WT    | WT    | MET amplification      | 2.309    | >10        |
|                 | BT-474    | AD                 | 2.7   | 32.0  | WT    | WT    | HER2 amplification     | 0.002    | 1.207     |
|                 | SK-BR-3   | AD                 | 2.7   | 23.0  | WT    | WT    | HER2 amplification     | 0.002    | 0.251     |
|                 | BT-474    | AD                 | 2.9   | 32.0  | WT    | WT    | HER2 amplification     | 0.002    | 0.251     |

AD, adenocarcinoma; LC, large cell carcinoma; SQ, squamous cell carcinoma.

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**Fig. 2.** Afatinib inhibits both HER2-amplified and HER2-mutant non-small-cell lung cancer and breast cancer cells. (a) HER2-amplified and HER2-mutant non-small-cell lung cancer cells were treated with afatinib or gefitinib for 72 h and IC\(_{50}\) values were determined using an MTS assay. Error bars indicate standard deviations. (b) Cells were treated with the indicated concentrations of afatinib for 6 h and lysates were subjected to Western blot analysis with the indicated antibodies. EGFR, epidermal growth factor receptor.
In previous preclinical studies, Perera et al.\(^9\) reported the efficacy of afatinib and the combination of afatinib with mTOR inhibitor rapamycin in HER2-mutant A775insYVMA transgenic mice or H1781 xenografts. As for other EGFR/HER2-TKIs, neratinib (HKI-272) or dacomitinib (PF00299804) showed efficacy in lung cancer cells with HER2 amplification or mutation in vitro\(^{(35–37)}\). In this study, we showed that afatinib efficiently inhibited growth of both HER2-amplified and HER2-mutant lung cancer cells as well as that of EGFR-mutant lung cancer cells at clinically achievable concentrations\(^{(38)}\) through G1 arrest and apoptotic cell death. In addition, we showed afatinib inhibited HER2 phosphorylation in HER2-mutant cells in the transmembrane domain. These facts were confirmed in BEAS-2B cells with various HER2 mutants transfection. Although the rate of HER2 mutations in the transmembrane domain is rare, afatinib may be a therapeutic option in patients with this alteration.

Regarding the HER2 mutations in lung cancer, reported mutations are concentrated in exon 20 and the majority of these mutations are A775insYVMA, G776VC, G776LC, G776C/V777insCG, and P780insGSP except one mutation, L755S, in exon 19\(^{(15,39)}\) and two transmembrane domain mutations, V659E and G660D, in exon 17.\(^{(16)}\) According to the COSMIC database (http://cancer.sanger.ac.uk/cosmic), A775insYVMA, G776VC, G776LC, and P780insGSP mutations account for 74% of all HER2 mutations in lung cancers. In addition, mutations in the αC-β4 loop (M774–R784) of the HER2 kinase domain accounted for 81% of HER2 mutations at the time of our survey.
on October 12, 2015. As mentioned, the 3-D structure of the modeled HER2–afatinib complex shows that the major mutations of HER2 are concentrated in an exposed area (A775–P780) of the αC–β4 loop, which is located on the back side of the ATP-binding pocket. Thus, these mutations are considered not to directly inhibit the binding of afatinib to the HER2 kinase domain. These facts, with our experimental results, support the assertion that afatinib is effective for the majority of HER2 mutations.

Although EGFR-mutant NSCLCs initially respond to the antitumor effects of EGFR–TKIs, most of these tumors eventually acquire resistance to the treatment. Several mechanisms of resistance have been reported, and the secondary T790M mutation in exon 20 of EGFR is the most common.40,41 In addition, Takezawa et al.42 recently reported HER2 amplification as a novel mechanism of acquired resistance to reversible EGFR-TKIs in patients with NSCLCs harboring EGFR mutations. Considering the findings we obtained, afatinib may be useful for overcoming irreversible EGFR-TKI resistance acquired through HER2 amplification.

In conclusion, we showed the antitumor effect of afatinib in lung cancers harboring HER2 alterations. Our results suggest that afatinib is a therapeutic option as a HER2-targeted therapy for NSCLC harboring HER2 amplification or mutations.

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Disclosure Statement
The authors have no conflict of interest.

Abbreviations
ALK anaplastic lymphoma kinase
EGFR epidermal growth factor receptor
HER2 human epidermal growth factor receptor 2
NSCLC non-small-cell lung cancer
PARP poly(ADP-ribose) polymerase
TKI tyrosine kinase inhibitor
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