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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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Key words

Afatinib, ERBB2, HER2, HER2-targeted therapy, non-small-cell lung cancer

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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 G_1 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 *KRAS*, *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]

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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% CO_2 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 pIDT-SMART (C-TSC) vector, pCMViR-TSC.[21] Transient transfection of the BEAS-2B cells with the mammalian expression vectors was carried out using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

Three-dimensional structure analysis. A 3-D structure model of HER2–afatinib was made by copying afatinib coordinates to the substrate binding pocket of a HER2 kinase domain structure (Protein Data Bank [PDB; http://www.pdb.org/] ID, 3RCD) from a superimposed EGFR–afatinib complex structure (PDB ID, 4G5J). The HER2 mutation sites of exon 20 reported in previous papers were mapped on the modeled complex structure. The figure was prepared using PyMOL (Schrödinger, New York, NY, USA).

Secondary structure assignment for the HER2 kinase domain structure was carried out with STRIDE to determine the residue numbers at the beginning and end of the αC–β4 loop of the HER2 kinase domain.[22]

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) (Merck 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).

DNA extraction. Genomic DNA was obtained from the cell lines with a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

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), as previously reported.[23] In brief, *LINE-1* was used as a reference gene for each copy number analysis. The dosage of each target and reference gene was calculated by the standard curve method. The relative copy number of each sample was determined by comparing the ratio of the target gene to *LINE-1* in each sample with the ratio of these genes in standard genomic DNA (Merck, Darmstadt, Germany).

Cell growth inhibition assay. Cells were cultured with or without drugs for 72 h and the sensitivities to the drugs were determined using a modified MTS assay with CellTiter 96 Aqueous One Solution Reagent (Promega, Fitchburg, WI, USA), as previously described.[24] The antiproliferative activities of each drug are shown as IC_{50}, 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. Doublets, 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 (5×10^6) 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 $V = 1/2 \times [(\text{shortest diameter})^2 \times (\text{the longest diameter})]$. When the tumors exceeded approximately 50 mm^3, 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]

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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 6 h. We then analyzed the HER2 signaling pathway 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 reports[19,25] 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 IC_{50} 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 IC_{50} values of 0.140 and 0.086 μM, respectively. Afatinib also showed strong antiproliferative effects on H1781 cells, with an IC_{50} of 0.039 μM. Regarding the sensitivity to gefitinib among *HER2*-altered cells, H2170 and H1781 cells, were resistant to gefitinib ($IC_{50} > 3$ μM). In contrast, Calu-3 cells were partially sensitive to gefitinib, with an IC_{50} of 0.471 μM, which was consistent with a previous report.[26] Furthermore, HCC827 cells (*EGFR*-mutant) were sensitive to afatinib with IC_{50} values of 0.001 μM, and H1975 cells (*EGFR*-L858R/T790M mutant) were moderately resistant to afatinib with IC_{50} 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 ($IC_{50} > 2$ μM). In addition, afatinib showed antiproliferative effects on *HER2*-amplified breast cancer cell lines (SK-BR-3 and BT-474 cells), with an IC_{50} 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-G_1 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 G_1 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 G_1 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.[9,27] One week after transplantation, when tumors exceeded approximately 50 mm^3, 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-

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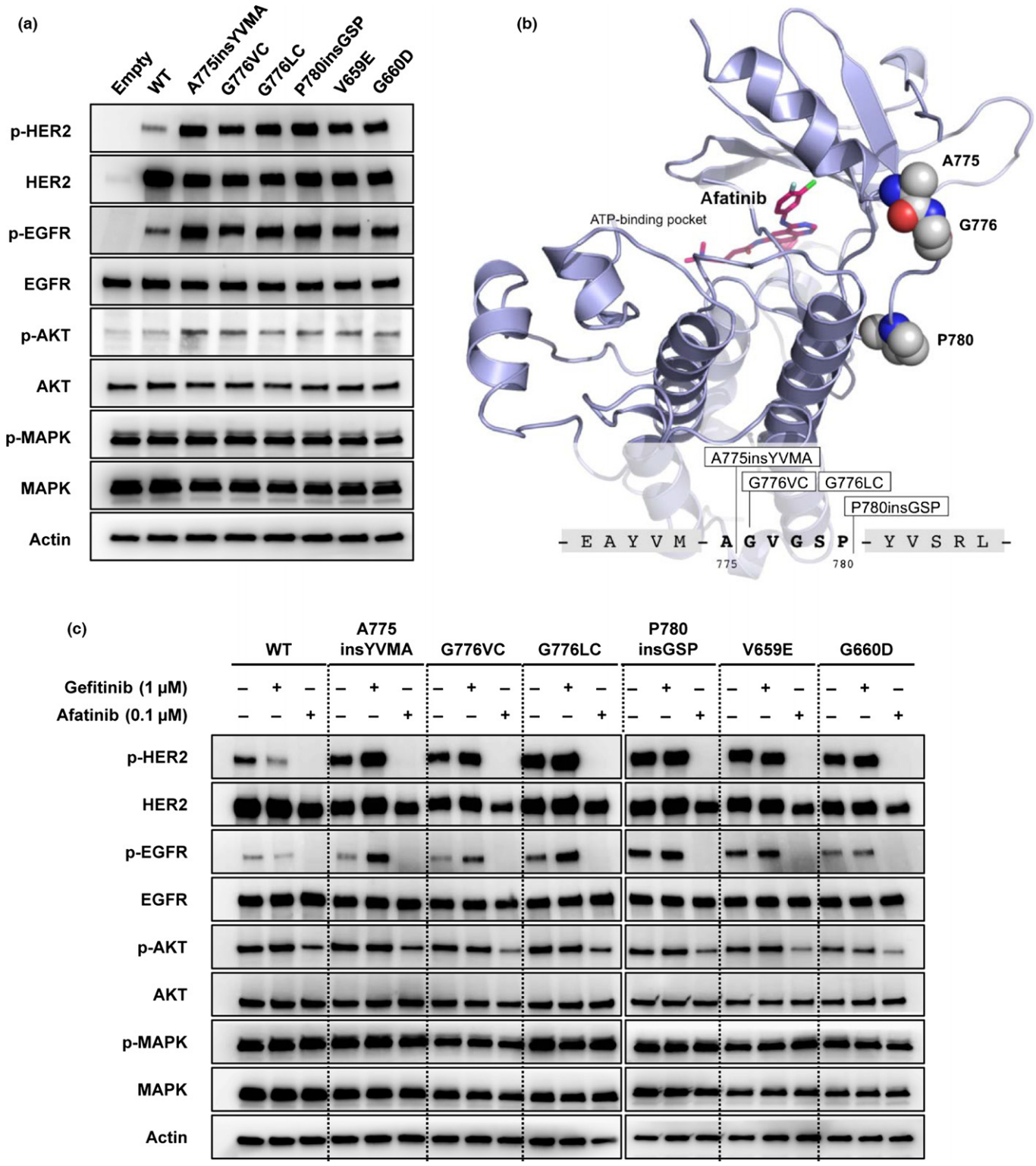

Fig. 1. Overexpressing wild-type or mutant *HER2* activates human epidermal growth factor receptor 2 (HER2) signaling, and afatinib inhibits them. (a) BEAS-2B cells were transiently transfected with wild-type HER2, its variants, A775insYVMA, G776VC, G776LC, P780insGSP, G660D, and V659E, or vector control. 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.

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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	G776VC	*HER2* mutation	0.039	3.565
	HCC827	AD	48.7	2.3	Exon 19 del(E746–A750)	WT	*EGFR* mutation	0.001	0.045
	H1975	AD	3.9	2.5	L858R, T790M	WT	*EGFR* mutation	0.713	>10
	A549	AD	2.8	3.1	WT	WT	*K-ras* mutation	5.275	>10
	H1299	LC	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
Breast cancer	SK-BR-3	AD	2.7	23.0	WT	WT	*HER2* amplification	0.002	1.207
	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.

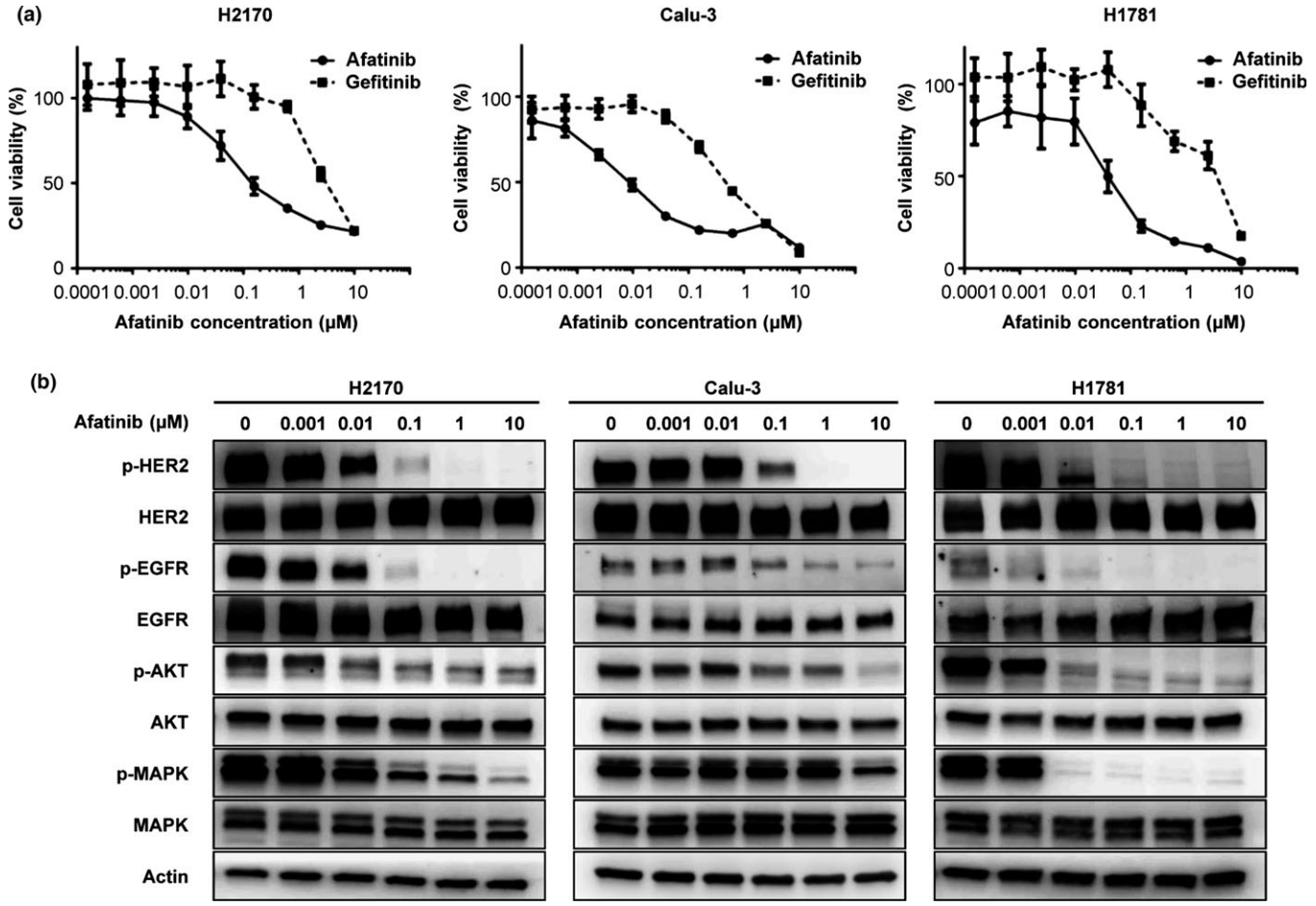

Fig. 2. Afatinib inhibits both *HER2*-amplified and *HER2*-mutant non-small-cell lung cancer and breast cancer cells. (a) *HER2*-amplified and *HER2*-mutant non-small-cell lung cancer cells were treated with afatinib or gefitinib for 72 h and IC_{50} values were determined using an MTS assay. Error bars 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.

histochemistry analysis.[30–32] 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.

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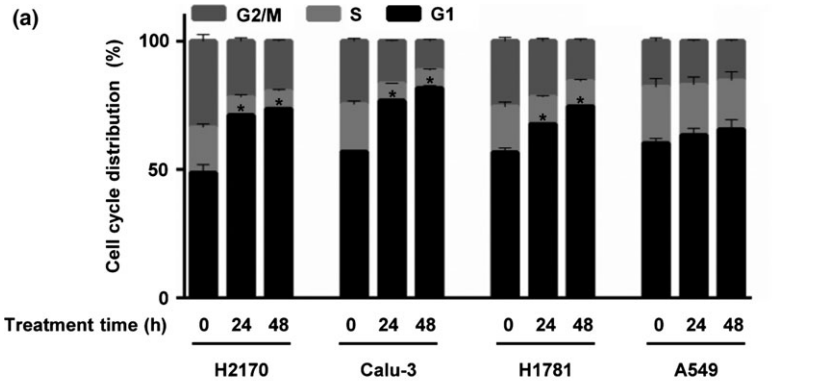

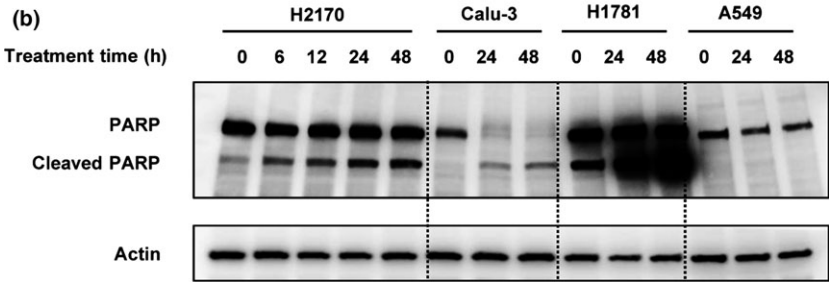

Fig. 3. Afatinib induces cell cycle arrest and apoptosis in *HER2*-dependent lung cancer cells. (a) Non-small-cell lung cancer cells were treated with 0.1 μM afatinib for 24 or 48 h and subjected to cell cycle analysis using flow cytometry. The graph represents the mean percentages of each phase in live cells ± SD of triplicate cultures. *$P < 0.05$ versus vehicle controls by one-way ANOVA followed by Dunnett's test. (b) Lysates from non-small-cell lung cancer cells were collected at the indicated time points after the addition of 0.1 μM afatinib and subjected to Western blot analysis with the indicated antibodies. PARP, poly(ADP-ribose) polymerase.

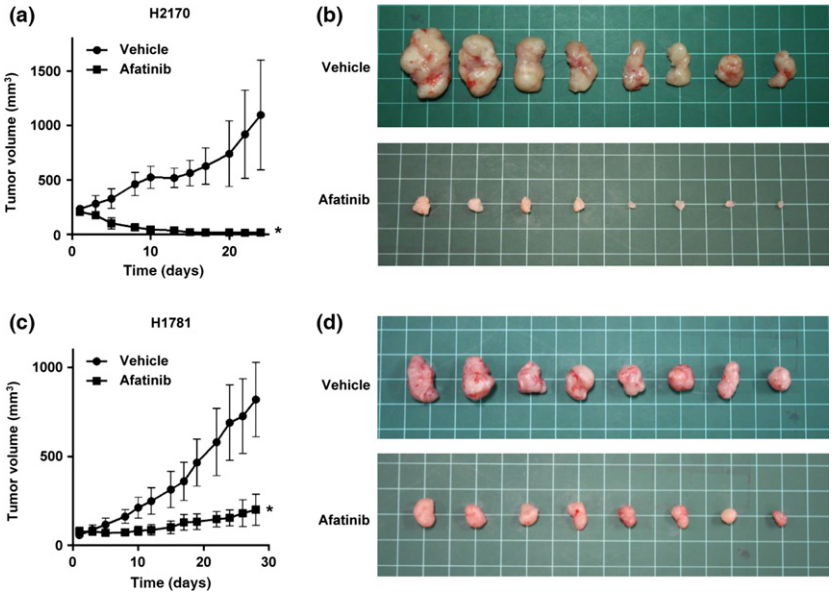

Fig. 4. Afatinib shows a strong antitumor effect on tumor growth in xenograft mouse model of *HER2*-altered lung cancer cells. (a) Mice with H2170 tumors were given vehicle or afatinib. Tumor volume was determined at the indicated days after the onset of treatment. Data represent mean ± SE ($n = 8$). *$P < 0.05$ versus vehicle controls by two-way ANOVA for repeated measurements. (b) Appearance of H2170 tumors after treatment at the time the mice were killed. (c) Mice with H1781 tumors were given vehicle or afatinib and analyzed as in (a). Data represent mean ± SE ($n = 8$). *$P < 0.05$ versus vehicle controls by two-way ANOVA for repeated measurements. (d) Appearance of H1781 tumors after treatment at the time the mice were killed.

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 G_1 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

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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 reversible 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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