

**Afatinib prolongs survival compared to gefitinib in an epidermal growth factor
receptor-driven lung cancer model**

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Abstract

An irreversible ErbB family blocker is expected to inhibit tumors with activating epidermal growth factor receptor (EGFR) mutations more strongly than reversible EGFR tyrosine kinase inhibitors and to overcome acquired resistance to the T790M secondary mutation. Eleven-week-old transgenic mice with *Egfr* exon 19 deletion mutation were treated with afatinib, gefitinib, or vehicle for 4 weeks. All mice were sacrificed at 15 weeks of age, and the number of superficial left lung tumors with a long axis exceeding 1 mm was counted. The afatinib-treated group had significantly fewer tumors than the vehicle group ($P < 0.01$) and tended to have fewer tumors than the gefitinib-treated group ($P = 0.06$). Pathologically, gefitinib-treated mice had clearer, more nodular tumors than afatinib-treated mice. Immunoblotting showed that afatinib suppressed not only pEGFR but also pHER2, and induced apoptosis for longer periods than gefitinib. Subsequently, when each drug was administered 5 days per week until death, afatinib significantly enhanced mouse survival compared with gefitinib (median survival time: 456 days versus 376.5 days; logrank test, $P < 0.01$). Finally, the combination of afatinib with bevacizumab was found to be superior to either drug alone in exon 19 deletion/T790M and L858R/T790M xenograft tumors. Overall, afatinib was more potent than gefitinib in tumors harboring an exon 19 deletion mutation, and the combination of afatinib with bevacizumab efficiently suppressed tumors harboring the

T790M secondary mutation.

Introduction

The selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib have shown clinical responses in patients with advanced non-small cell lung cancer (NSCLC), especially in those with tumors harboring activating *EGFR* mutations (1-3). Five prospective randomized clinical trials showed that gefitinib and erlotinib as initial treatments for EGFR-mutant NSCLC improved progression-free survival compared with chemotherapy (4-8). Most of the mutations in these studies were a deletion in exon 19 or a point mutation in exon 21 (L858R).

Following erlotinib treatment, patients with an exon 19 deletion showed significantly improved response rate, progression-free survival, and overall survival compared with those with the L858R mutation (9). Recently, Won et al. summarized the differences in clinical benefits between the two mutation types using data from 12 studies (10) and suggested that patients with an exon 19 deletion might benefit more from gefitinib or erlotinib treatment compared with those with the L858R mutation.

Regardless of having the deletion or L858R mutation, the majority of patients with EGFR-mutant NSCLC are initially sensitive to EGFR TKI, but subsequently become resistant to it (11). In such patients, approximately half have tumors with a secondary

T790M mutation in exon 20 of *EGFR* (12-15) and around 20% have tumors with amplifications of the *MET* gene (16-18). For the former group, irreversible second-generation EGFR inhibitors such as afatinib have been introduced. Afatinib was shown to be effective in tumor cells and a transgenic mouse model with an L858R point mutation with or without a T790M secondary mutation (19-22). Although the effectiveness of this agent for tumor cells with an EGFR exon 19 deletion mutation was shown *in vitro* (23), no preclinical studies using transgenic mice have yet been reported. Moreover, a study by Sos et al. found limited efficacy of afatinib monotherapy in T790M-mutated cells (24). To overcome this, the combination of EGFR TKI with a monoclonal antibody has been evaluated; for example, afatinib with an EGFR-specific antibody such as cetuximab, and gefitinib with a vascular endothelial growth factor-specific antibody such as bevacizumab (22, 25).

In this study, we investigated the *in vitro* efficacy of afatinib against tumor cells with activating EGFR mutations with or without the T790M mutation and evaluated it in combination with bevacizumab against xenograft tumors harboring the T790M mutation. In addition, we compared the effectiveness of afatinib with that of gefitinib in EGFR transgenic mice.

Materials and Methods

Cell culture and growth inhibition assay *in vitro*

The pulmonary adenocarcinoma cell lines PC-9 and H1975 were purchased from Immuno-Biological Laboratories (Takasaki, Gunma, Japan) and from the American Type Culture Collection (Manassas, VA), respectively. PC-9 was derived from an untreated Japanese patient with pulmonary adenocarcinoma carrying an in-frame deletion in *EGFR* exon 19 (delE746-A750) (26). H1975 was derived from a female never smoker patient with pulmonary adenocarcinoma carrying exon 21 missense mutation (L858R) and exon20 missense mutation (T790M) (27). Gefitinib-resistant RPC-9 cells were established in our laboratory by continuously exposing PC-9 cells to gefitinib (14). PC-9 cells were sensitive to EGFR TKI, while RPC-9 cells carrying delE746-A750 with T790M and H1975 cells carrying L858R with T790M were EGFR TKI-resistant. All the cell lines used were authenticated.

Cells were cultured at 37°C with 5% CO₂ in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Growth inhibition was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (28). Cells were placed on 96-well plates at a density of 2,000 per well and continuously exposed to each drug for 96 h. MTT was then added to each well, and the cells were

incubated for 4 hours at 37°C before measurement of absorbance at 570 nm of cell lysate by a microplate reader (Bio-Rad, Model 680, USA). Absorbance values were expressed as a percentage of that for untreated cells. The drug concentration required to inhibit the growth of tumor cells by 50% (IC₅₀) was used to evaluate the effect of the drug. Each assay was performed in triplicate.

Reagents and antibodies

Afatinib (Fig. 1A) was kindly provided by Boehringer Ingelheim (Mannheim, Germany). Gefitinib and bevacizumab were purchased from Everlth Co., Ltd. (Okayama, Japan). Rabbit antisera against EGFR, phospho-specific (p) EGFR (pY1068), human epidermal growth factor receptor (HER)2, pHER2 (pY877), pHER3 (pY1289), mitogen-activated protein kinase (MAPK), pMAPK (pT202/pY204), Akt, pAkt (pSer473), PARP, cleaved caspase 3, GAPDH, and β-actin were purchased from Cell Signaling Technology (Tokyo, Japan). Anti-HER3 monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

Immunoblotting analysis

Cells or frozen tissue were lysed in radioimmunoprecipitation assay buffer [1% Triton

X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β -glycerolphosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate-containing protease inhibitor tablets (Roche Applied Sciences GmbH, Mannheim, Germany). Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with Enhanced Chemiluminescence Plus (GE Healthcare Biosciences, Tokyo, Japan).

Animal husbandry and drug treatment

We generated transgenic mice constitutively expressing the delE748-A752 version of mouse *Egfr* (29) and the L858R version of human *EGFR* (30) in the lung only using the SP-C promoter, which is exclusively activated in type 2 alveolar epithelial cells. The transgenic mice had no distant metastases (29, 30). All animals were kept under pathogen-free conditions with abundant food and water as specified in the guidelines of the Department of Animal Resources, Okayama University Advanced Science Research Center, Japan. Afatinib (5 mg/kg) or gefitinib (5 mg/kg) were given once daily, 5 days per week as a suspension by gavage. Afatinib was prepared in 0.5 w/v% methyl cellulose and gefitinib was prepared in 1% polysorbate 80 by homogenization and

ball-milled with glass beads for 24 h. After 4 weeks administration of each drug, the mice were sacrificed by cervical dislocation and the lungs excised and the numbers of tumors on the surface of the left lungs were counted. The right lungs were used for protein extracts. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research.

Histology

Animals were sacrificed by cervical dislocation and the lungs, the skins, the intestines and the livers were excised. The organs were inflated with 10% paraformaldehyde in phosphate-buffered saline (PBS), fixed in 10% paraformaldehyde overnight at room temperature, embedded in paraffin, and sectioned every 5 μm (West Japan Pathology, Okayama, Japan). The sections were stained with hematoxylin-eosin.

Xenograft model

Female athymic mice at 7 weeks of age were purchased from Japan Charles River Co. All mice were provided with sterilized food and water and housed in a barrier facility under a 12-h light/dark cycle. Cells (2×10^6) were injected s.c. into the backs of the

mice. When the tumor volume exceeded 100 mm³, the mice were randomly assigned to one of five groups (5 to 6 mice per group) that received either vehicle, 10 mg/kg afatinib, 5 mg/kg bevacizumab, 10 mg/kg afatinib plus 5 mg/kg bevacizumab or 10 mg/kg gefitinib plus 5 mg/kg bevacizumab. Vehicle, gefitinib and afatinib were administered once daily, 5 days per week by gavage. Bevacizumab (5 mg/kg) was administered twice a week i.p. Tumor volume ($\text{width}^2 \times \text{length}/2$) was determined twice a week. Administration of each drug was continued during the experimental term (53 days for RPC-9 cells and 40 days for H1975 cells).

Statistical analysis

Statistical analysis was performed using the SPSS Base System™ and Advanced Statistics™ programs (SPSS, Chicago, IL). Group differences were compared using Student's *t*-test. Survival time was defined as the period from initiation of treatment to death or last follow-up evaluation. Overall survival curves were calculated using the Kaplan–Meier method. $P < 0.05$ was considered statistically significant.

Results

Afatinib suppressed PC-9 and RPC-9 cells at lower concentrations than gefitinib

The mean IC₅₀ ± standard error (SE) of afatinib for PC-9 cells and RPC-9 cells was

0.00032 ± 0.00016 μmol/L and 0.164 ± 0.07784 μmol/L, respectively, which was less than that of gefitinib for PC-9 (0.0033 ± 0.01074 μmol/L) and RPC-9 cells (11.45 ± 2.77717 μmol/L) (Fig. 1B). pEGFR, pAKT, and pERK in PC-9 cells were suppressed by both treatment with afatinib (1 nmol/L) and with gefitinib (100 nmol/L) (Fig. 1C). In RPC-9 cells, afatinib was 100-fold less active (100 nmol/L) than in parental cells, while gefitinib was ineffective (10,000 nmol/L) (Fig. 1D). In addition, 1 nmol/L and 100 nmol/L of afatinib completely inhibited pHER2 in PC-9 cells and RPC-9 cells, respectively (Fig. 1E). Interestingly, gefitinib also suppressed pHER2 in both cell types at concentrations that could suppress pEGFR (Fig. 1E). These results suggest that afatinib was effective in PC-9 and RPC-9 cells at lower concentrations than gefitinib.

Afatinib may be more effective than gefitinib for treatment of lung cancer induced by the EGFR mutation in exon 19

We next investigated the effect of afatinib on lung tumors induced by the activating EGFR mutation *in vivo*. Eleven-week-old transgenic mice with an exon 19 deletion mutation were treated with oral afatinib (5 mg/kg/day), gefitinib (5 mg/kg/day), or vehicle alone from 11 to 15 weeks of age. No significant difference was found between the three groups with respect to weekly measurement of body weight (Fig. 2A). We

previously reported that no interstitial lung disease was found in the mice treated with gefitinib (29) and no other toxicities of skin, intestine and liver developed in the present study (Supplemental Fig. 1).

All mice were sacrificed at 15 weeks of age and the number of superficial left lung tumors with a long axis exceeding 1 mm was determined to be 0.12 ± 0.13 , 1.25 ± 0.60 , and 9.00 ± 1.5 in the afatinib-treated group, gefitinib-treated group, and vehicle group, respectively (Fig. 2B). This represented a significant difference between the afatinib-treated group and vehicle group ($P < 0.01$) and indicated a tendency for fewer tumors with afatinib than with gefitinib ($P = 0.06$). Pathologically, gefitinib-treated mice had clearer, more nodular tumors than afatinib-treated mice (Fig. 2C). We performed the same experiment using transgenic mice with the L858R EGFR exon 21 point mutation and observed no superficial tumors in gefitinib- or afatinib-treated mice and no toxicity (Fig. 2D and 2E). In addition, there was no clear tumor formation in pathological samples in any group (Fig. 2F). Toxicities in lung, skin, intestine and liver were not observed in the L858R transgenic mice treated with afatinib (Supplemental Fig. 1).

Afatinib suppressed EGFR and HER2 and induced apoptosis for longer than

gefitinib

Immunoblotting was performed to confirm the histological response at the protein level. The lungs of 11-week-old transgenic mice showed overexpression of both pEGFR and pHER2 (Fig. 3A), consistent with our previous data (29, 31). Subsequently, 14-week-old mice were sacrificed at the indicated time after single administration of each drug. In gefitinib-treated mice, pEGFR was suppressed at 2 and 6 h but was recovered at 10 h (Fig. 3B). On the other hand, in afatinib-treated mice, pEGFR was suppressed at 2, 6, and 10 h and was partially recovered at 14 and 18 h (Fig. 3B). At 14 h, pEGFR expression was higher in the gefitinib-treated mice and lower in the afatinib-treated mice than in mice without treatment. In the gefitinib-treated mice, pHER2 was decreased at 2 and 6 h but was recovered by 10 h (Fig. 3B), whereas in afatinib-treated mice, it was suppressed from 2 to 18 h (Fig. 3B). These results were comparable with those *in vitro* (Fig. 1E).

Cleaved PARP and cleaved caspase 3 were analyzed to examine the effects on apoptosis. This was induced at 6 and 10 h only in gefitinib-treated mice but was observed from 6 to 18 h in afatinib-treated mice (Fig. 3C). It might be due to the difference of duration of suppression of pEGFR and pHER2 in comparison with control (2 to 6 h versus 2 to 18 h). These results suggest that afatinib is more effective than

gefitinib because it suppresses not only pEGFR but also pHER2 and induces apoptosis over a longer period than gefitinib.

Effect of afatinib on the survival of transgenic mice

Short-term (4 weeks) administration of afatinib tended to be more effective than that of gefitinib in the lung cancer model induced by a deletion mutation in exon 19 (Fig. 2B, 2C). To investigate the efficacy of long-term afatinib treatment, we randomly assigned 7-week-old transgenic mice to receive vehicle alone (n=13), gefitinib (5 mg/kg/day, n=14), or afatinib (5 mg/kg/day, n=15) and orally administered each drug for 5 days per week until toxicity or death. All mice in the control group died, with a median survival time of 119 days. We also investigated change of the tumor sizes in transgenic mice after the treatment using computed tomography (Supplemental Fig. 2A). The tumors were responded to afatinib or gefitinib. Tumor reduction in afatinib-treated mice was more profound than that in gefitinib-treated mice (Supplemental Fig. 2B).

Survival curves of gefitinib-treated and afatinib-treated mice are shown in Fig. 4A. Afatinib treatment significantly enhanced the survival of transgenic mice (logrank test, $P < 0.01$), with a median survival time of 456 days compared with 376.5 days for gefitinib-treated mice (Fig. 4A). No toxic death was observed in any mice. Four weeks

after the initiation of treatment, body weight in the control group was significantly lower than in the gefitinib and afatinib groups (Fig. 4B). Gefitinib-treated mice lost a significant amount of weight compared with afatinib-treated mice after 34 weeks (Fig. 4B), which is likely to be a result of tumor progression.

Combination of afatinib with bevacizumab in T790M-mutant tumors

We hypothesized that the combination of afatinib with bevacizumab would be effective for treating tumors harboring the T790M mutation. Although afatinib or bevacizumab alone was ineffective against RPC-9 xenograft tumors (Fig. 5A), the combination of afatinib with bevacizumab inhibited tumor growth more efficiently. An H1975 xenograft tumor was then used to determine whether the superiority of the combination therapy could be confirmed in other cancer cells harboring the T790M mutation; again the drug combination proved to be effective (Fig. 5B). Subsequently, we compared the effect of gefitinib and afatinib when combined with bevacizumab in xenograft models of RPC-9 and H1975 (Supplemental Figure 3A, B). Actually, both combinations significantly suppressed tumor growth compared with vehicle alone (day 21 in RPC-9; days 5, 8, 12 and 15 in H1975). Although the tumor volume treated with bevacizumab and afatinib was consistently smaller than that treated with bevacizumab and gefitinib,

there were not significant differences in any points.

To examine why the combination of bevacizumab with afatinib was more effective than afatinib alone or bevacizumab alone (Fig. 5A), we analyzed the RPC-9 tumors by Western blotting. The expression levels of pEGFR, pAKT and cleaved caspase 3 were similar between afatinib alone and the combination of bevacizumab with afatinib (Supplemental Fig. 4A). Since bevacizumab alone did not affect expression of pEGFR, pAKT and cleaved caspase 3, we evaluated neovascularization using immunohistochemistry (Supplemental Fig. 4B). CD31-positive blood vessels in tumors treated by bevacizumab alone or combination of bevacizumab with afatinib were significantly reduced compared with tumors treated by vehicle or afatinib alone. These results suggested that the combination therapy might be effective because the suppression effect of neovascularization by bevacizumab was added to tumor growth inhibition by afatinib.

Discussion

Irreversible EGFR TKIs such as afatinib are expected to inhibit tumors with activating EGFR mutations more strongly than reversible EGFR TKIs and to overcome the acquired resistance attributable to the T790M secondary mutation. We found that afatinib was more potent than gefitinib in tumors harboring an exon 19 deletion

mutation and that the combination of afatinib with bevacizumab was useful in the treatment of tumors harboring the T790M secondary mutation.

Afatinib was effective at lower concentrations than gefitinib in PC-9 cells (Fig. 1B, 1C), although interestingly, the concentration of gefitinib (100 nmol/L) that could suppress pEGFR in PC-9 cells also inhibited pHER2 (Fig. 1E). This presumably reflects the temporary suppression of the EGFR/HER2 heterodimer by gefitinib. However, the ErbB family blocker afatinib suppressed pHER2 at a lower concentration (1 nmol/L) than gefitinib (Fig. 1E).

Subsequently, we investigated the efficacy of afatinib in a transgenic mouse model and found it to be more effective than gefitinib in the lung of exon 19 deletion mutant mice (Fig. 2C). Afatinib-treated mice also suppressed pEGFR and pHER2 for longer than gefitinib-treated mice, and afatinib appeared to induce apoptosis at 14 and 18 h despite partial recovery of the EGFR signal (Fig. 3C). Finally, afatinib significantly prolonged the survival of transgenic mice compared with gefitinib (Fig. 4A), indicating its higher potency in tumors with an EGFR exon 19 deletion mutation. We await the findings of the LUX-Lung 7 trial (NCT01466660), a phase IIb trial of afatinib versus gefitinib in the treatment of 1st line EGFR mutation-positive lung adenocarcinoma, to determine whether the data are correlated with our observations.

Afatinib was also shown to be effective at lower concentrations (by approximately 1/100th) than gefitinib in both parent and resistant tumor cell lines harboring the T790M secondary mutation, but it appeared that such tumor cells might be resistant to both afatinib and gefitinib. In fact, in a phase IIb/III randomized trial, 29 (7%) of 390 patients who initially failed reversible EGFR TKI treatment had a partial response to afatinib (32). Sos et al. reported a dramatic reduction in the potency to gatekeeper-mutated EGFR of irreversible EGFR TKIs compared with single activating EGFR mutations (24). The present results are consistent with this (Fig. 1), suggesting that irreversible EGFR TKI monotherapy is insufficient for overcoming resistance by gatekeeper mutations.

The combination of afatinib with an EGFR-specific antibody (cetuximab) was found to induce shrinkage of erlotinib-resistant tumors harboring the T790M mutation (22) as well as be effective in patients with the T790M mutation (33). Moreover, we previously showed *in vivo* that the combination of gefitinib with the vascular endothelial growth factor inhibitor bevacizumab inhibited the growth of tumors harboring the T790M mutation more efficiently than gefitinib alone (25). In the present study, we demonstrated that the combination of afatinib with bevacizumab in exon 19 deletion/T790M (RPC-9) and L858R/T790M (H1975) xenograft tumors was superior to

each drug alone.

This study had some limitations. Firstly, the effect of afatinib on tumorigenesis was not examined. We previously reported that the transgenic mice developed atypical adenomatous hyperplasia at age 4 weeks and multifocal adenocarcinoma of varying sizes at age 7 weeks (30). Administration of gefitinib to 3-week-old transgenic mice for 1 week before carcinogenesis reduced the amount of phosphorylated EGFR in the lungs of the mice to the baseline level. Gefitinib inhibited tumorigenesis completely in the mouse model. Although we expect afatinib may have chemopreventive effect, it remains unclear from our study. In the present study, 11-week-old transgenic mice were treated with oral afatinib, gefitinib, or vehicle alone from 11 to 15 weeks of age (Fig. 2). Meanwhile, to investigate the efficacy of long-term afatinib treatment, we randomly assigned 7-week-old transgenic mice to receive vehicle alone, gefitinib, or afatinib and orally administered each drug (Fig. 4). Since transgenic mice developed adenocarcinoma at age 7 weeks, we think this study showed successful treatment for lung cancer. From the point of view of chemopreventive effect of afatinib, we should administer the drug to 3-week old transgenic mice. Further study including analysis of molecular targets should be needed. Secondary, we could not show whether the drug doses used for mouse studies were clinically relevant. The clinical data including

recommended dose of afatinib were not available when we initiated this study. Now, we know afatinib was administered to human at the dose of 50 mg/day although the dose of gefitinib was 250 mg/day. In addition, the pharmacokinetics and pharmacodynamics in the mouse are usually different from those in human. Those are reasons why we administered the same doses of both drugs (10 mg/kg for xenograft mice; 5 mg/kg/day for transgenic mice).

In conclusion, afatinib was more potent than gefitinib in the treatment of lung adenocarcinoma with an EGFR exon 19 deletion mutation. Moreover, afatinib in combination with bevacizumab may overcome the resistance attributed to the T790M secondary mutation.

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Figure legends

Figure 1. Effects of gefitinib and afatinib on the growth of PC-9 and RPC-9 cells *in vitro*.

A, Drug structures of afatinib and gefitinib. B, Drug sensitivity. Cells were seeded (2,000 per well) on 96-well plates and were cultured for 96 h in complete medium containing various concentrations of gefitinib and afatinib, after which cell viability was assessed as described in Materials and Methods. *Points*, mean values of triplicate cultures; *Bars*, SE. C, D, Effects of each drug on the phosphorylation status of EGFR and its downstream signaling in (C) PC-9 and (D) RPC-9 cells. Cells were incubated with various concentrations of each drug for 2 h and cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated or total forms of EGFR, STAT3, AKT or ERK as well as with those to β -actin. E, Inhibition of HER2. PC-9 and RPC-9 cells were incubated with various concentrations of each drug for 2 h and cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated or total forms of HER as well as with those to β -actin.

Figure 2. Effects of gefitinib or afatinib in transgenic mice.

A, Changes in body weight in each group. Transgenic mice with *Egfr* exon 19 deletion

mutation were administered each drug 5 days per week for 4 weeks. Body weight was measured once a week. *Bars*, SE. B, Number of superficial left lung tumors with the long axis exceeding 1 mm in each group. Tumor numbers were counted after 4 weeks administration of each drug. *Bars*, SE. *, $p < 0.01$, for vehicle versus either gefitinib or afatinib. C, The left lung after 4 weeks administration of each drug. The sections were stained with hematoxylin-eosin. D, Changes in body weight in each group. Transgenic mice with *EGFR* exon 21 L858R mutation were administered each drug 5 days per week for 4 weeks. Body weight was measured once a week. *Bars*, SE. E, Number of superficial left lung tumors with the long axis exceeding 1 mm in each group. Tumor numbers were counted after 4 weeks administration of each drug. *Bars*, SE. *, $p < 0.01$, for vehicle versus either gefitinib or afatinib. F, The left lung after 4 weeks administration of each drug. The sections were stained with hematoxylin-eosin.

Figure 3. Comparison of gefitinib and afatinib in the suppression of pEGFR and pHER2 in transgenic mice with an exon 19 deletion mutation.

A, Protein levels in the lungs of untreated 14-week-old transgenic (Tg) and control mice (WT) examined by immunoblotting. Each 14-week-old mouse was sacrificed and proteins were extracted from the right lung. GAPDH was used as a loading control.

Phosphorylated or total forms of EGFR, HER2 and HER3 were examined. B, Protein levels in the right lungs of 14-week-old mice sacrificed at the indicated points. GAPDH was used as a loading control. Phosphorylated or total forms of EGFR, HER2 and were examined. C, Expression of the apoptosis-related proteins cleaved PARP and cleaved caspase 3 examined by immunoblotting in 14-week-old mice sacrificed at 2, 6, 10, 14, and 18 h after single administration of each drug.

Figure 4. Long-term effects of gefitinib or afatinib in transgenic mice.

A, Survival curves of vehicle (solid line), gefitinib-treated (dotted line) and afatinib-treated (broken line) mice. + indicates the censored mouse. B, Change in body weight of vehicle, gefitinib-treated and afatinib-treated mice. *Bars*, SE. *, $p < 0.05$, for vehicle versus either gefitinib or afatinib (left) and gefitinib versus afatinib (right).

Figure 5. Xenograft model.

A, B, Growth curves of (A) RPC-9 and (B) H1975 xenograft tumors in animals receiving oral administration of 10 mg/kg/d afatinib 5 times per week or i.p. administration of 5 mg/kg bevacizumab twice a week. Difference in tumor volume was compared using Student's *t* test. *Bars*, SE. *, $p < 0.05$, for the combination of afatinib

with bevacizumab versus other treatments (vehicle, afatinib alone, and bevacizumab alone).