rat IgG (Cell Signaling Technology, Danvers, MA) for 40 min. Peroxidase activity was visualized with DAB reactions. The sections were counterstained with hematoxylin.

Statistical analysis

Between-group differences were analyzed by one-way ANOVA. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, San Diego, CA); $p < 0.05$ was considered statistically significant.

Results

BEZ235 suppressed the viability of *EGFR* mutant lung cancer cells in the presence of exogenous HGF

PC-9 and HCC827 cells were highly sensitive to erlotinib, whereas exogenously added HGF induced erlotinib resistance (Fig. 1*a*). BEZ235, a dual inhibitor of PI3K and mTOR, suppressed the viability of these cell lines in a dose-dependent manner. Under these experimental conditions, exogenously added HGF did not decrease the sensitivity of these cell lines to BEZ235, suggesting that BEZ235 may have the potential to overcome HGF-triggered resistance to EGFR–TKIs in *EGFR* mutant lung cancer cells.

BEZ235 suppressed the phosphorylation of Akt and its downstream molecules even in the presence of HGF

To explore the molecular mechanism by which BEZ235 suppressed cell viability, even in the presence of HGF, we examined the expression and phosphorylation status of the proteins EGFR and Met and their downstream molecules (Erk1/2, Akt and p70S6K) in PC-9 cells (Fig. 1*b*). In the absence of HGF, erlotinib inhibited the phosphorylation of EGFR and Erk1/2 markedly and Akt and p70S6K slightly. Although BEZ235 did not affect the phosphorylation of EGFR or Erk1/2, it markedly inhibited the phosphorylation of Akt and p70S6K. In the presence of HGF, erlotinib inhibited EGFR phosphorylation, but did not inhibit the phosphorylation of Met, Erk1/2, Akt and

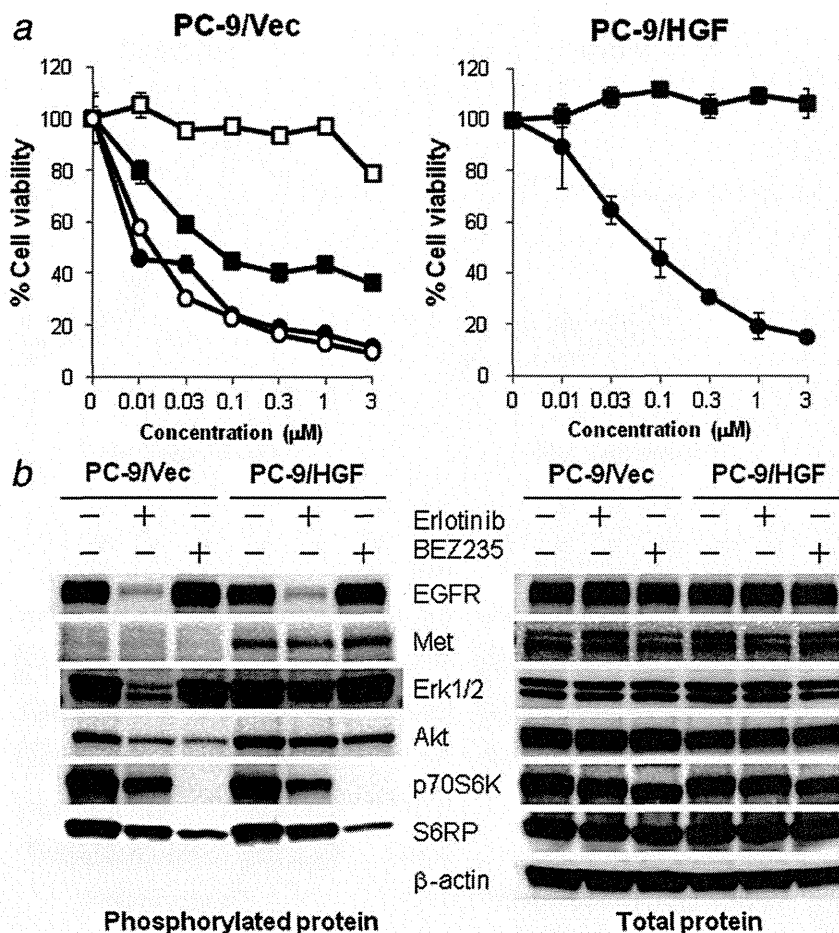

Figure 2. BEZ235 overcame erlotinib resistance caused by endogenous HGF. (*a*) PC-9/Vec and PC-9/HGF cells (2×10^3 cells/well) were incubated with various concentrations of drugs and/or HGF (20 ng/mL) (■: erlotinib, □: erlotinib + HGF, ●: BEZ235, O:BEZ235 + HGF). Bars show SD. (*b*) PC-9/Vec and PC-9/HGF cells were treated with or without erlotinib (0.3 μM) or BEZ235 (0.3 μM) for 4 hr. The cell lysates were harvested and subjected to Western blotting. The data shown are representative of three independent experiments with similar results.

p70S6K. Although BEZ235 did not affect the phosphorylation of EGFR, Met or Erk1/2, it inhibited the phosphorylation of Akt and p70S6K. These results suggested that BEZ235 inhibited the viability of *EGFR* mutant lung cancer cells primarily by inhibiting the PI3K/mTOR (Akt/p70S6K) pathway, even in the presence of HGF.

BEZ235 suppressed the viability of *EGFR* mutant lung cancer cells in the presence of endogenous HGF

We previously showed that, in patients with *EGFR* mutant lung cancer and acquired resistance to EGFR–TKIs, HGF was present primarily in cancer cells, suggesting that autocrine HGF may be crucial roles for EGFR–TKI resistance in lung cancer patients.[31] To further explore the effect of BEZ235 on autocrine HGF-triggered resistance to EGFR–TKIs, we generated stable HGF-gene transfectants in PC-9 cells (PC-9/HGF); as a control, we generated PC-9 cells transfected with vector alone (PC-9/Vec). PC-9/HGF cells secreted high concentrations of HGF (27.8 ± 0.9 ng/48 hr), whereas the concentrations of HGF secreted by PC-9 and PC-9/Vec cells were under the limit of detection (data not shown). In addition, PC-9/HGF cells became resistant to erlotinib. We found that BEZ235 inhibited the viability of both PC-9/Vec and PC-9/HGF cells in a dose-dependent manner (Fig. 2*a*), whereas BEZ235 did not induce apoptosis (Supporting Information Fig. 1), in the line of the previous studies.[26] These results suggest that BEZ235 circumvented resistance induced by both endogenous and exogenous HGF.

We also examined the expression and phosphorylation status of the proteins EGFR, Met and their downstream

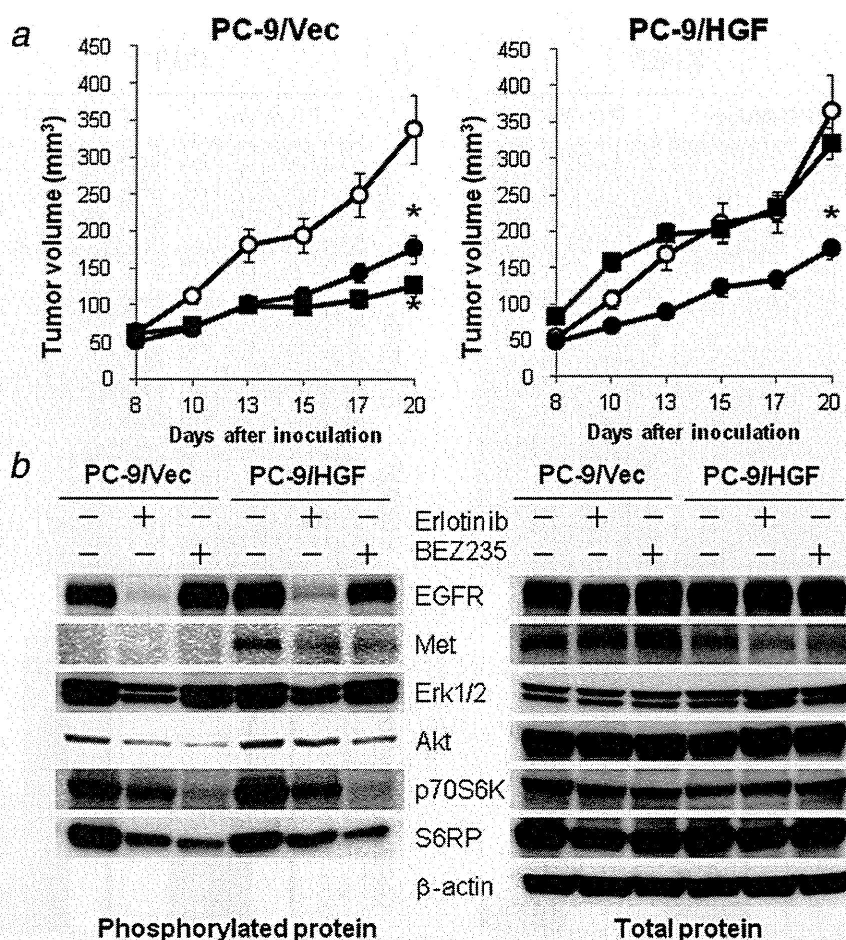

Figure 3. BEZ235 treatment inhibited the growth of erlotinib-resistant PC-9/HGF tumors in SCID mice. (*a*) SCID mice bearing PC-9/Vec or PC-9/HGF tumors were administered 25 mg/kg erlotinib or 20 mg/kg BEZ235 once daily from day 8 to day 20 (○: control, ■: erlotinib, ●: BEZ235). Tumor volume was measured using calipers on the indicated days. Mean ± SE tumor volumes are shown for groups of four to five mice. $^*p < 0.01$ (one-way ANOVA). (*b*) SCID mice with PC-9/Vec or PC-9/HGF tumors were administered erlotinib 25 mg/kg and/or BEZ235 20 mg/kg daily for 4 days. Four hours after final administration, tumors were harvested and the relative levels of proteins in the tumor lysates were determined by Western blotting. The data shown are representative of two independent experiments with similar results.

molecules (Erk1/2, Akt and p70S6K) in PC-9/Vec and PC-9/HGF cells by Western blotting (Fig. 2*b*). PC-9/Vec cells expressed EGFR and Met proteins, with only EGFR being discernibly phosphorylated, as well as expressing the downstream proteins Erk1/2, Akt, p70S6K and S6RP, all of which were phosphorylated. Erlotinib inhibited the phosphorylation of EGFR, Erk1/2, Akt, p70S6K and S6RP. BEZ235 did not affect the phosphorylation of EGFR, Met or Erk1/2, but inhibited the phosphorylation of Akt slightly and p70S6K and S6RP, the downstream molecules of mTOR, markedly. In PC-9/HGF cells, Met was also phosphorylated presumably owing to HGF produced in an autocrine manner. Erlotinib inhibited the phosphorylation of EGFR, but not of Met, Erk1/2, Akt or p70S6K. In contrast, BEZ235 did not inhibit the phosphorylation of EGFR, Met or Erk1/2, but inhibited the phosphorylation of Akt slightly and p70S6K and S6RP markedly. These results indicate that BEZ235 suppresses the phosphorylation of downstream molecules of mTOR, irrespective of the presence or the absence of endogenous HGF.

BEZ235 suppressed the growth of erlotinib-resistant tumors induced by HGF *in vivo*

We next evaluated whether BEZ235 could overcome HGF-induced resistance to erlotinib *in vivo*. Oral administration of erlotinib or BEZ235 markedly suppressed the growth of PC-9/Vec tumors (Fig. 3*a*). In contrast, erlotinib failed to inhibit the growth of PC-9/HGF-tumors, indicating that HGF induced resistance to erlotinib *in vivo*. Under these experimental conditions, BEZ235 markedly suppressed the growth of PC-9/HGF tumors, indicating that BEZ235 may be useful for controlling the growth of HGF-induced erlotinib-resistant tumors. We also assessed the molecular mechanisms by which BEZ235 inhibited the growth of PC-9/HGF tumors. Western

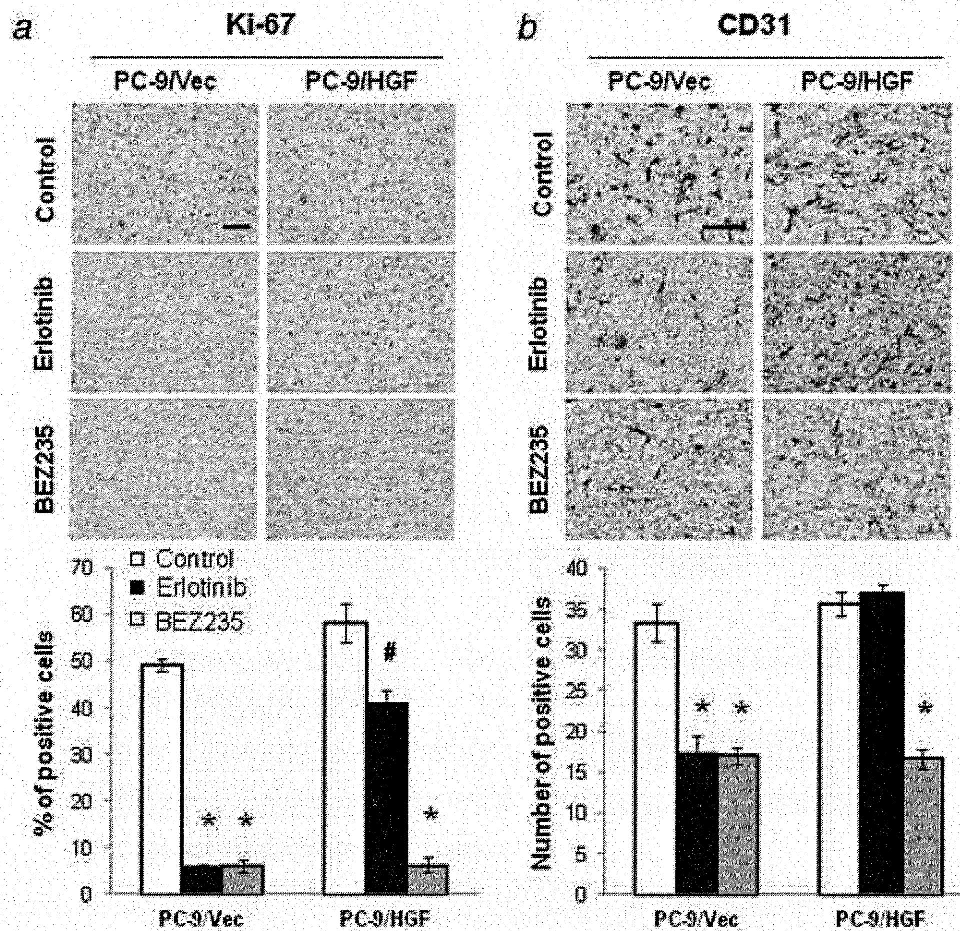

Figure 4. BEZ235 treatment inhibited tumor-cell proliferation and angiogenesis. SCID mice with PC-9/Vec or PC-9/HGF tumors were administered erlotinib 25 mg/kg and/or BEZ235 20 mg/kg daily for 4 days. Four hours after final administration, tumors were harvested and tumor cell proliferation (*a*: Ki-67, bar: 50 μm) and angiogenesis (*b*: CD31, bar: 100 μm) were determined by immunohistochemistry. Lower panes show quantification of positive cells. $^{\#}p < 0.01$, $^{*}p < 0.001$ (one-way ANOVA). Bars show standard errors. The data shown are representative of two independent experiments with similar results. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

blot analyses showed that erlotinib markedly inhibited the phosphorylation of EGFR and Erk1/2 and slightly inhibited the phosphorylation of Akt and p70S6K in PC-9/Vec tumors (Fig. 3*b*). In PC-9/HGF tumors, Met was phosphorylated, whereas erlotinib markedly inhibited the phosphorylation of EGFR and slightly inhibited the phosphorylation of Met, Erk1/2, Akt and p70S6K. Although BEZ235 had no effect on the phosphorylation of EGFR or Erk1/2, it markedly inhibited the phosphorylation of Akt and p70S6K. These results indicated that the antitumor effects of BEZ235 on the growth of PC-9/HGF cells may be owing primarily to its inhibition of the PI3K/mTOR (Akt/p70S6K) pathway.

We also examined tumor cell proliferation (Ki-67), apoptosis (TUNEL) and angiogenesis (CD31) by immunohistochemistry. Although erlotinib treatment induced apoptosis in PC-9/Vec tumors but not PC-9/HGF tumors, BEZ235 treatment did not induce apoptosis in PC-9/Vec or PC-9/HGF tumors (Supporting Information Fig. 2). Erlotinib treatment of animals with PC-9/Vec tumors markedly inhibited tumor cell proliferation and angiogenesis, whereas erlotinib treatment of mice with PC-9/HGF tumors inhibited cell proliferation only marginally and had no effect on angiogenesis (Fig. 4). In contrast, treatment with BEZ235 markedly inhibited cell proliferation and angiogenesis of both PC-9/Vec and PC-9/HGF tumors. These findings suggest that BEZ235 inhibited the growth of PC-9/HGF tumors, at least in part, by inhibiting angiogenesis.

To further explore the antiangiogenic influence of BEZ235, we examined its effect on tumor cell production of vascular endothelial growth factor (VEGF), a prominent proangiogenic molecule. Both PC-9 and PC-9/Vec cells constitutively produced detectable levels of VEGF, which were stimulated by HGF (Fig. 5*a*). Consistent with these observations, PC-9/HGF cells produced higher levels of VEGF than did PC-9 and PC-9/Vec cells. BEZ235 suppressed VEGF production in these cancer cells in the presence or the absence of HGF. We also assessed the direct effect of BEZ235 on the viability of endothelial cells *in vitro*. BEZ235 did not inhibit constitutive viability of HMVEC cells in medium with 10% FBS alone (Fig. 5*b*). VEGF increased the viability of HMVECs and BEZ235 inhibited this increase in viability in a dose-dependent manner.

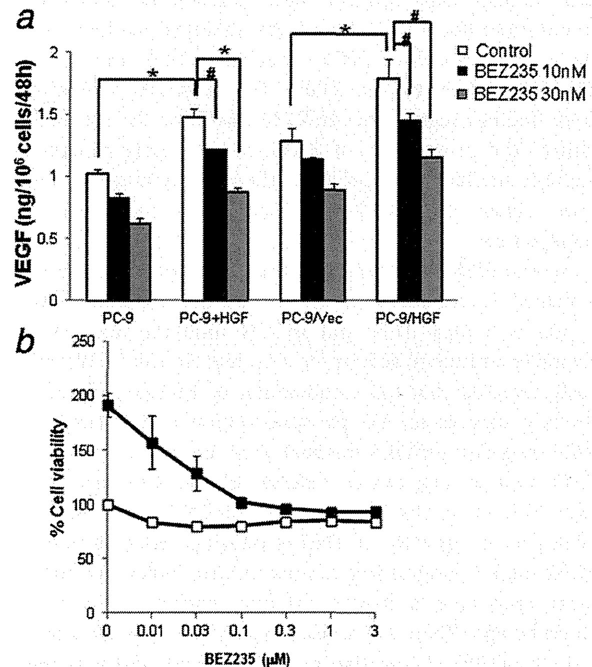

Figure 5. BEZ235 inhibited VEGF production by cancer cells and endothelial viability. (*a*) Tumor cells were incubated with or without HGF (50 ng/mL) in the presence of different concentrations of BEZ235 for 48 hr. Then, supernatants were harvested and the number of tumor cells was counted. VEGF concentration in the supernatants was determined by ELISA (R&D Systems). VEGF levels corrected for tumor cell number are shown. $^{\#}p < 0.05$, $^{*}p < 0.001$. (*b*) HMVECs were incubated in RPMI-1640 medium with 10% FBS (control), or RPMI-1640 medium with 10% FBS plus VEGF (50 ng/mL) with different concentrations of BEZ235 for 72 hr. Then, cell viability was determined using the MTT assay. Bars show SD. The data shown are from two independent experiments with similar results.

Discussion

We have shown here that BEZ235, a dual inhibitor of PI3K and mTOR that is being evaluated in clinical trials, could suppress the growth of *EGFR* mutant lung cancer cells without remarkable apoptosis induction, irrespective of the presence of HGF. Our observations suggest that BEZ235, even as monotherapy, may be useful for the management of patients with *EGFR* mutant lung cancer and HGF-induced EGFR–TKI resistance.

Activation of the PI3K/Akt and mitogen-activated protein kinase/Erk pathways has been shown crucial for the survival and growth of *EGFR* mutant lung cancer cells.[29,32,33] We found that BEZ235 markedly inhibited the phosphorylation of Akt, a downstream molecule of PI3K, and p70S6K, a downstream molecule of mTOR, but not Erk1/2, *in vitro* and *in vivo*. Moreover, although BEZ235 did not induce apoptosis of PC-9 cells, it inhibited cell number increase of PC-9 cells (Supporting Information Fig. 3), indicating that BEZ235 made PC-9 cells become quiescent. These results strongly suggest that the antitumor effect of BEZ235 may be owing primarily to its inhibition of PI3K and mTOR.

The antitumor effect of BEZ235 may also be owing, at least in part, to its antiangiogenic activity. BEZ235 may act on both cancer cells and endothelial cells to suppress angiogenesis.[34] BEZ235 inhibits PI3K/mTOR signaling in tumor cells, thereby suppressing their production of VEGF. BEZ235 also inhibits PI3K/mTOR signaling in endothelial cells, suppressing their proliferation and permeability.[35] We recently reported that HGF activates the Met/PI3K/Akt pathway and facilitates not only EGFR–TKI resistance but also angiogenesis *via* VEGF production in *EGFR* mutant lung cancer cells.

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Our findings here confirm these observations and further demonstrate that BEZ235 markedly inhibited angiogenesis in an *in vivo* model of HGF-producing *EGFR* mutant lung tumors resistant to the EGFR–TKI erlotinib. Collectively, these findings suggest that BEZ235 may have the potential to control the progression of *EGFR* mutant lung cancers *via* multiple mechanisms, including the inhibition of the resistance signal to EGFR–TKI and the suppression of angiogenesis.

Several PI3K inhibitors have been developed and are being evaluated in clinical trials against various types of tumors. PI-103 is a dual PI3K and mTOR inhibitor that revealed favorable antitumor activity *in vitro* and *in vivo*.[36] We previously reported that the combination of PI-103 and gefitinib strongly suppressed Akt phosphorylation and circumvented HGF-triggered gefitinib resistance in an *in vivo* model of *EGFR* mutant lung cancer, whereas PI-103 alone had no activity. We have shown here that BEZ235 alone markedly inhibited the growth of HGF-producing, erlotinib-resistant *EGFR* mutant lung cancer tumors *in vivo*, indicating that this agent may have a higher potential relative to PI-103 as monotherapy. There are at least explanations for the superior activity of BEZ235 relative to PI-103. First, the two agents differ in anti-mTOR activity although both have similar IC_{50}s for PI3Ks.[26,28,37] In our experiments with HGF-gene transfectants, a PI-103 concentration of >0.3 μM was required to discernibly inhibit the phosphorylation of p70S6K, a downstream molecule of mTOR, whereas much lower concentrations of BEZ235 were required (Supporting Information Fig. 4A). Similarly, 0.3 μM BEZ235 was more effective than 1 μM PI-103 in terms of inhibition of PC-9-cell viability (Supporting Information Fig. 4B). Recent studies also reported that the concentration of BEZ235 required to inhibit the growth of breast cancer cell lines with a *KRAS* or *PIK3CA* mutation was about 100-fold lower than the concentration of PI-103.[38] Moreover, the addition of the mTOR inhibitor rapamycin to PI-103 markedly inhibited Akt-Ser473 phosphorylation, thereby suppressing cell growth.[39] We also found that the single use of GDC-0941 (a selective PI3K inhibitor) or rapamycin inhibited the viability of PC-9 cells by about 40% and their combined use further inhibited cell viability, irrespective of the presence of HGF. Moreover, combined use of rapamycin and PI-103 also further inhibited cell viability, just like BEZ235 alone (Supporting Information Fig. 4B). These results indicate the importance of the combined inhibition of PI3K and mTOR. The second reason for the difference between BEZ235 and PI-103 may be owing to the differences in drug metabolism and pharmacodynamics. PI-103 is rapidly metabolized in cells, with a half life of 0.7–1.3 hr,[37] whereas BEZ235 potently inhibited Akt-Ser473 as late as 16 hr after *in vivo* administration.[28] Therefore, BEZ235 may inhibit target activity in tumor cells more durably than PI-103, and may thereby be more effective than PI-103.

BEZ235 is being evaluated in clinical trials in various types of tumors, including lung cancer. Several trials have shown the safety of this compound when given as monotherapy. When 400 mg of BEZ235 was given twice daily (800 mg/day) in solid tumors, Cmax reached more than 7,000,ng/mL.[40] Therefore, the doses of BEZ235 we used in our study seem to be clinically relevant and the findings presented here suggest that BEZ235 may control *EGFR* mutant lung cancer even after they develop resistance to EGFR-TKIs. Clinical evaluation of BEZ235 in patients with EGFR-TKI-resistant *EGFR* mutant lung cancer is warranted.

Acknowledgements

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ORIGINAL ARTICLE

Akt kinase-interacting protein1, a novel therapeutic target for lung cancer with *EGFR*-activating and gatekeeper mutations

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Despite initial dramatic response, epidermal growth factor receptor (EGFR) mutant lung cancer patients always acquire resistance to EGFR-tyrosine kinase inhibitors (TKIs). Gatekeeper T790M mutation in *EGFR* is the most prevalent genetic alteration underlying acquired resistance to EGFR-TKI, and *EGFR* mutant lung cancer cells are reported to be addictive to EGFR/Akt signaling even after acquired T790M mutation. Here, we focused on Akt kinase-interacting protein1 (Aki1), a scaffold protein of PI3K (phosphoinositide 3-kinase)/PDK1 (3-phosphoinositide-dependent protein kinase)/Akt that determines receptor signal selectivity for non-mutated *EGFR*, and assessed its role in *EGFR* mutant lung cancer with or without gatekeeper T790M mutation. Cell line-based assays showed that Aki1 constitutively associates with mutant *EGFR* in lung cancer cells with (H1975) or without (PC-9 and HCC827) T790M gatekeeper mutation. Silencing of *Aki1* induced apoptosis of *EGFR* mutant lung cancer cells. Treatment with *Aki1* siRNA dramatically inhibited growth of H1975 cells in a xenograft model. Moreover, silencing of *Aki1* further potentiated growth inhibitory effect of new generation EGFR-TKIs against H1975 cells *in vitro*. Aki1 was frequently expressed in tumor cells of *EGFR* mutant lung cancer patients (53/56 cases), including those with acquired resistance to EGFR-TKI treatment (7/7 cases). Our data suggest that Aki1 may be a critical mediator of survival signaling from mutant *EGFR* to Akt, and may therefore be an ideal target for *EGFR* mutant lung cancer patients, especially those with acquired EGFR-TKI resistance due to *EGFR* T790M gatekeeper mutation.

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Keywords: Akt kinase-interacting protein1; *EGFR* mutation; novel therapeutic target; lung cancer

INTRODUCTION

Lung cancer with epidermal growth factor receptor (EGFR)-activating mutations, such as exon 19 deletion and exon 21 L858R point mutation, responds to the EGFR-tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib.[1] Recent clinical trials demonstrated much longer progression-free survival for EGFR mutant lung cancer patients when treated with gefitinib compared with conventional chemotherapy.[2,3] However, almost without exception, the responders relapse after various times due to acquiring resistance to EGFR-TKIs.[1,4]

The development of gatekeeper mutations, such as T315I in Abl,[5] D473H in SMO[6] and L1196M in ALK,[7] is the most common mechanism of acquired TKI resistance.[8] In cases of *EGFR* mutant lung cancer, *EGFR* T790M mutation is detected in about 50% of patients with acquired resistance to EGFR-TKIs.[4,8,9] T790M mutation results in increased EGFR affinity to adenosine triphosphate, reducing binding of EGFR-TKIs, and thus inducing resistance.[10] However, *EGFR* mutant lung cancer cells with T790M mutation are still dependent on EGFR-mediated signaling,[10] and therefore further elucidation of mutant EGFR-mediated signaling may facilitate the development of novel effective therapeutic strategies against lung cancer with *EGFR* mutations, including T790M gatekeeper mutation.

New generation EGFR-TKIs, such as irreversible EGFR-TKIs and mutant EGFR selective TKIs, were expected to overcome acquired resistance caused by T790M secondary mutation.[11-15] However, several irreversible EGFR-TKIs failed to meet primary end points in clinical trials in EGFR-TKI-refractory lung cancer and induced severe adverse effects, such as diarrhea, skin rush/acne, stomatitis and nail effect.[1,16] More recently, a phase Ib trial of EGFR dual inhibition with irreversible EGFR-TKI afatinib plus anti-EGFR monoclonal antibody cetuximab indicated with a 40% objective response rate in 47 patients with EGFR-TKI acquired resistance,[17] suggesting that many tumors are still addicted to the EGFR signaling pathway, including *EGFR* T790M gatekeeper mutation in clinical trials. Therefore, new intensification treatment targeting EGFR signaling is expected to get for more clinical benefit, whereas the feasibility of these strategies should be evaluated carefully in clinical trials.

Receptor tyrosine kinases, such as EGFR, PDGFRs and VEGFRs, utilize several common downstream signaling pathways, including MAPK/ERK and PI3K/Akt, while each receptor shows different or specific biological activity after ligand stimulation. Scaffold proteins that can simultaneously interact with two or more protein binding partners are thought to ensure specificity as well as temporal regulation of signal transduction. Thus, scaffold

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proteins may be important targets for regulating receptor-mediated signaling. There is accumulating evidence that Akt signaling is essential for mediating survival signals in *EGFR* mutant lung cancer cells.[18,19] Although several molecules, including KSP,[20] Paxillin,[21] RKIP,[22] and JIP-1,[23] are known to act as scaffolds for MAPK-ERK,[24] scaffold proteins for Akt have not been well documented. Recently, we reported Akt kinase-interacting protein1 (Aki1) as the first identified scaffold in the PI3K/PDK1/Akt pathway. Aki1 selectively forms a complex with EGFR and Akt in response to EGF stimulation, mediates Akt activation by PDK1, and hence contributes to cell survival and proliferation.[25] However, Aki1, the scaffold proteins for therapeutic target in cancers, have yet to be identified.

In the present study, we examined whether Aki1 would act as a determinant of receptor signaling selectivity of mutant EGFR and could be a therapeutic target for *EGFR* mutant lung cancer, including that with T790M gatekeeper mutation.

RESULTS

High levels of Aki1 protein expression in *EGFR* mutant lung cancer cell lines

As the first step to assess the involvement of Aki1 in EGFR-mediating signal of lung cancer cells, we examined the expression of Aki1 protein and its associated proteins (PDK1, Akt and EGFR) in five human lung adenocarcinoma cells with or without *EGFR* mutations, comparing that in two human lung embryonic fibroblast cell lines, by western blotting (Figure 1a). All of the cell lines examined expressed Aki1 and PDK1 protein at various levels. The levels of Aki1 tend to be higher in EGFR mutant lung cancer cell lines than in lung fibroblast cell lines.

EGFR was also detected in all lung cancer and fibroblast cell lines at various levels. Interestingly, phosphorylated EGFR was detected in EGFR mutant lung cancer cell lines, but not detected in EGFR wild-type lung cancer cell lines and fibroblast cell lines. The co-detection of Aki1 and phosphorylated EGFR in these cell lines suggested interactions between Aki1 and mutant EGFR because Aki1 was shown to bind preferentially to activated wild-type EGFR.[25]

Aki1 constitutively associates with *EGFR* without ligand stimulation in *EGFR* mutant lung cancer cells

To determine the role of Aki1 in the EGFR/PDK1/Akt pathway, we examined the association between Aki1 and EGFR by immunoprecipitation. Aki1 constitutively associated with EGFR in all three *EGFR* mutant lung cancer cell lines (Figure 1b). Consistent with the results of previous studies,[25] Aki1 did not associate with IGF-1 R, irrespective of IGF-1 stimulation, indicating selective binding of Aki1 to EGFR (Figure 1b). Moreover, treatment with EGFR-TKI did not affect the association between Aki1 and EGFR/PDK1/Akt (Supplementary Figure S1). These results further suggest that Aki1 may be involved deeply in signal transduction through mutant EGFR.

Specific downregulation of *Aki1* inhibits cell viability and induces cell apoptosis in *EGFR* mutant lung cancer cells

To determine the role of Aki1 in *EGFR* mutant lung cancer cell lines, we used specific small interfering RNA (siRNA) for *Aki1* knockdown. Treatment with *Aki1*-specific siRNA suppressed Aki1 protein expression, and more decreased the viability of *EGFR* mutant cells (PC-9, HCC827 and H1975) than *EGFR* wild-type cells (A549 and PC14PE6) (Figure 2a and Supplementary Figure S2). To confirm the specificity of the *Aki1* siRNA used, we constructed RNA interference (RNAi)-resistant *Aki1* cDNA by mutating the sequence targeted by *Aki1* siRNA without changing the amino acid sequence. Transfection of wild-type *Aki1* or RNAi-resistant *Aki1* resulted in increased expression of Aki1 protein in PC-9 cells (Supplementary Figure S3A). Treatment with *Aki1* siRNA attenuated Aki1 protein expression and cell viability in parental and even in wild-type *Aki1*-transfected cells (Supplementary Figures S3B and C). However, *Aki1* siRNA did not downregulate exogenous Aki1 in RNAi-resistant *Aki1* cDNA-transfected cells. Transfection of RNAi-resistant *Aki1* cDNA overcame the *Aki1* siRNA-mediated decrease in cell viability (Supplementary Figure S3C), indicating the specificity of siRNA to *Aki1*. On the other hand, the effects of *Aki* siRNA in lung fibroblasts, MRC-5 and IMR-90, were only marginal (Figure 2a), suggesting that Aki knockdown selectively inhibits viability of cancer cells with dependent EGFR signal, especially in *EGFR* mutant lung cancer cells. In addition, to rule out any bystander effect of the siRNA, we performed cell culture using two color labeling. We found that *Aki1*-1 siRNA did not show any discernible bystander effect. Therefore, we conclude that the bystander effect is not the primary mechanism by which the *Aki1*-1siRNA treatment inhibited tumor cell growth, under our experimental conditions (Supplementary Figure S5A and B). Therefore, we focused solely on EGFR mutant lung cancer cells. Western blotting analyses indicated that *Aki1* knockdown reduced phosphorylation of downstream molecules, Akt and S6, and increased the levels of the proapoptotic molecule, cleaved PARP (poly (ADP-ribose) polymerase; Figure 2b), consistent with the decrease in cell viability. Furthermore, we also found that knockdown of *Aki1* discernibly induced apoptosis in PC-9, HCC827 and H1975 cells (Figure 2c).

We next assessed the effect of Aki1 inhibition, in comparison with EGFR inhibition, in *EGFR* mutant lung cancer cell lines. Like *EGFR* knockdown and erlotinib, *Aki1* knockdown considerably inhibited viability of PC-9 and HCC827 cells with exon 19 deletion in *EGFR*. In addition, *Aki1* knockdown inhibited viability of H1975 cells with exon 21 L858R and exon 20 T790M double mutations as potently as *EGFR* siRNA, whereas erlotinib had no effect (Figures 3a and b). These results suggest that targeting of Aki1 may be valuable for treating *EGFR* mutant lung cancer cells, especially with T790M gatekeeper mutation.

Aki1 knockdown inhibits tumor growth of lung cancer with EGFR T790M secondary mutation *in vivo*

Next, we examined the antitumor potential of *Aki1* siRNA against H1975 cells with *EGFR* T790M gatekeeper mutation *in vivo*. Intratumoral injection of either scramble or *Aki1* siRNA complexed with invivofectamine was performed on days 5 and 8. In a previous report, *MAGE-D1* gene knockdown by three direct injections of siRNA complicated with invivofectamine into the local region indicated 50% inhibition of protein expression.[26] *Aki1* siRNA treatment dramatically inhibited tumor growth in comparison with control or scramble siRNA (Figures 4a and b). We confirmed knockdown of Aki1 and the inhibition of downstream signaling molecule, S6, in tumors by western blotting (Figure 4c). These results clearly indicated the therapeutic potential of *Aki1* siRNA against lung cancer with *EGFR* T790M mutation *in vivo*.

Combined Aki1 and *EGFR* blockade strongly suppressed cell viability of lung cancer cells with *EGFR* T790M secondary mutation

Irreversible EGFR-TKIs and mutant selective EGFR-TKIs were developed to overcome *EGFR* T790M gatekeeper mutation-mediated resistance to erlotinib and gefitinib. Here, we examined whether Aki1 knockdown could augment the therapeutic efficacy of these new generation EGFR-TKIs. Irreversible EGFR-TKI, CL-387,785 and BIBW2992, and the mutant-selective EGFR-TKI, WZ4002, reduced the viability of H1975 cells, whereas erlotinib had no such effect. *Aki1* knockdown suppressed cell viability and further augmented the various dose inhibitory effects of CL-387,785, BIBW2992 and WZ4002 (Figure 5a and Supplementary Figure S4). Consistent with these findings, *Aki1*

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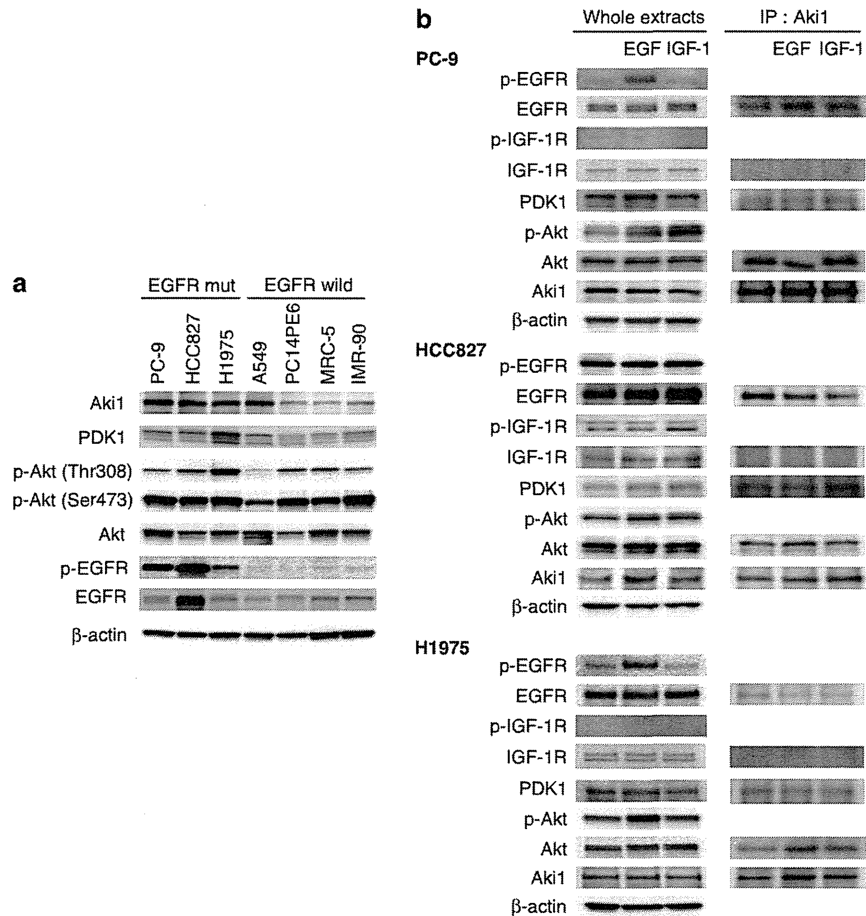

Figure 1. Aki1 expression and association to EGFR in EGFR mutant human lung cancer cell lines. (**a**) *EGFR* mutant human lung cancer cell lines (PC-9, HCC827 and H1975), *EGFR* wild-type human lung cancer cell lines (A549 and PC14PE6) and human lung fibroblast cell lines (MRC-5 and IMR-90) were lysed and the indicated proteins were detected by western blotting. (**b**) *EGFR* mutant lung cancer cell lines were treated with or without EGF (50 ng/ml) or IGF-1 (50 ng/ml) for 10 min. Then, cells were lysed and the indicated proteins were detected by western blotting with or without immunoprecipitation of Aki1.

knockdown decreased the levels of Akt and S6 phosphorylation, and increased the level of Par-4 and cleaved PARP, when combined with WZ4002 (Figure 5b). These results suggest the usefulness of *Aki1* knockdown combined with new generation EGFR-TKIs against lung cancer with *EGFR* T790M gatekeeper mutation.

Aki1 is frequently expressed in *EGFR* mutant lung cancer

We next examined Aki1 expression in 56 clinical specimens obtained from 56 lung cancer patients with *EGFR* mutation (Figure 6a and Supplementary Table S1). To confirm the specificity of the Aki1 by immunohistochemical staining, we performed Aki1 antibody absorption test by Aki1 peptide. The staining of Aki1 was remarkably diminished by pretreatment of sections with an Aki peptide at 4 °C overnight, compared with saline treatment, indicating the specificity of the antibody which we used for Aki1 staining (Supplementary Figure S6). Forty-two tumors were obtained from EGFR-TKI naive patients. Seven tumors were from patients who showed intrinsic resistance to the EGFR-TKIs, gefitinib or erlotinib. Another seven tumors were from patients who showed acquired resistance to EGFR-TKIs (Figure 6a). Of 42 EGFR-TKI naive tumors, the presence of Aki1 protein was scored as 2+ in 31 tumors (74%), 1+ in 8 tumors (19%), and – in 3 tumors (7%). Aki1 protein was detected diffusely in all of seven tumors with intrinsic resistance: 2+ in 4 (57%), 1+ in 3 (43%). Aki1 protein was detected diffusely in all of seven tumors with acquired resistance: 2+ in 6 (86%), 1+ in 1 (14%) (Figure 6a). Aki1 was detected in all tumors with acquired resistance, including four tumors with *EGFR* T790M mutation (Supplementary Table S1). These findings suggest involvement of Aki1 in EGFR-mediating signaling in lung cancer with *EGFR* mutations, including T790M gatekeeper mutation.

DISCUSSION

The results of the present study indicated that Aki1 constitutively associates with mutant EGFR even in the presence of EGFR-TKI. Silencing of *Aki1* induces apoptosis of *EGFR* mutant lung cancer cells, indicating that Aki1 has crucial roles in survival signal transduction in lung cancer cells with *EGFR* mutations. In a xenograft model, silencing of *Aki1* markedly inhibited growth of lung cancer cells with *EGFR* T790M gatekeeper mutation. Furthermore, Aki1 was frequently expressed in tumor cells of *EGFR* mutant lung cancer patients. Notably, it was detected in all tumors with acquired resistance to gefitinib or erlotinib, suggesting that Aki1 is an ideal target for *EGFR* mutant lung cancer, especially in cases with acquired EGFR-TKI resistance due to *EGFR* T790M gatekeeper mutation.

Although Aki1 associates with wild-type EGFR when activated by EGF,[25] it binds constitutively with mutant EGFR (Figure 1b).

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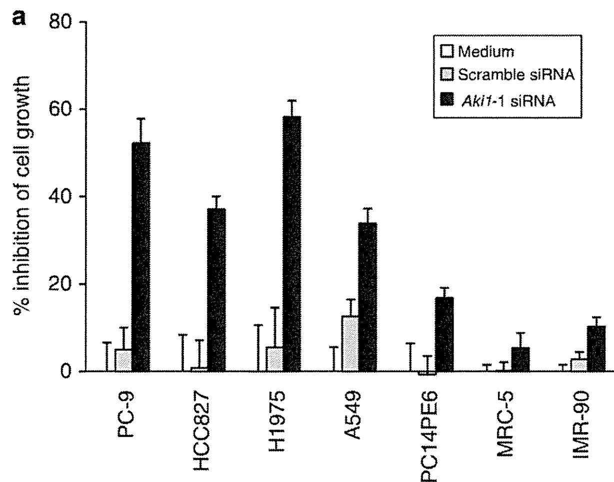

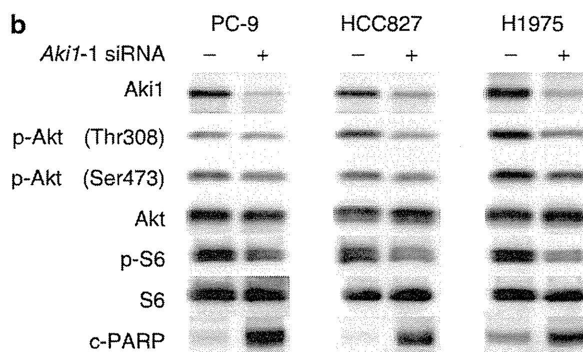

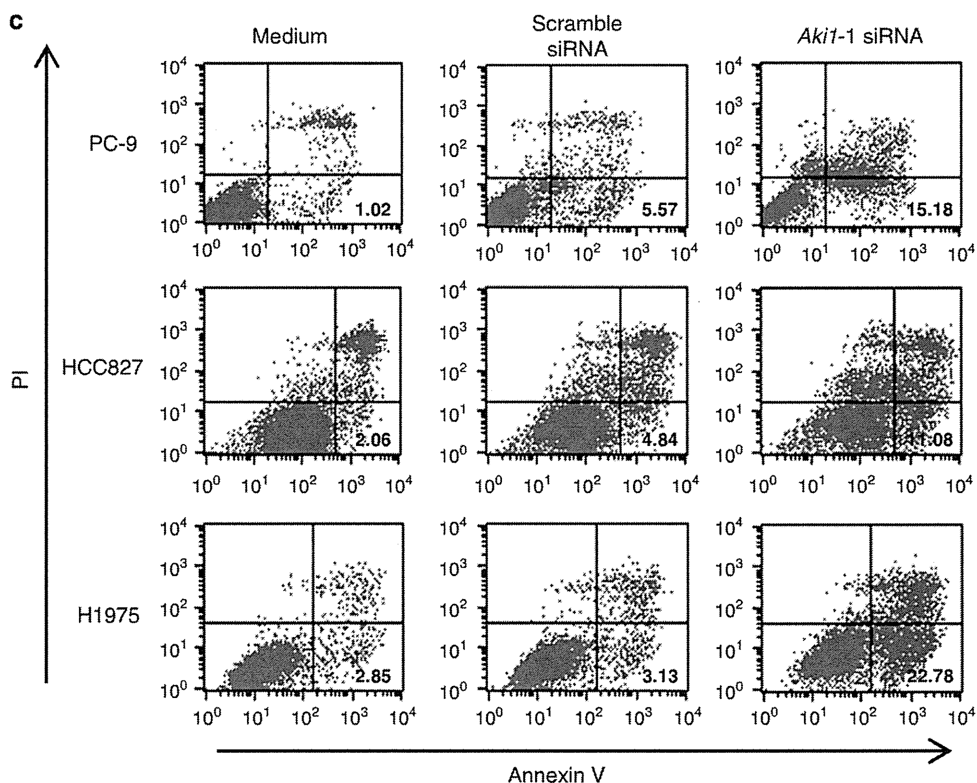

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Previous studies indicated enhanced kinase activity and transformation capabilities of EGFR in the presence of L858R or exon 19 deletion mutation.[27,28] Crystal structure analysis of the L858R mutant EGFR showed that this substitution activates the kinase through disruption of autoinhibitory interactions, resulting in receptors with high kinase activity compared with wild-type EGFR.[10,29,30] Consistent with these observations, mutant EGFR was constitutively phosphorylated, while the levels were varied among cell lines used in the present study. Taken together, these results indicate that Aki1 binds constitutively with mutant EGFR because mutant EGFR is constitutively activated.

There is accumulating evidence that scaffold proteins maintain signaling specificity and facilitate the activation of pathway components.[24,31,32] We showed that Aki1 constitutively forms complexes with EGFR, PDK, and Akt in *EGFR* mutant lung cancer cells. As in EGFR non-mutated cancer cells,[25] Aki1 did not bind to IGF-1 R even after stimulation with IGF-1 in *EGFR* mutant cells, indicating that Aki1 is the determinant of receptor signaling selectivity for EGFR. In a phase II clinical trial, anti-IGF-1 R antibody improved the response rate of conventional chemotherapy in non-small-cell lung cancer.[33] However, the phase III trial was terminated because of a trend toward poorer overall survival in the group with anti-IGF-1 R antibody. A preliminary report of toxicity from a phase II trial with the anti-IGF-1 R antibody demonstrated severe adverse events, including hyperglycemia.[34] These findings indicated difficulty of targeting IGF-1 R in cancer. As Aki1 is an EGFR-selective scaffold protein, Aki1 inhibition may have advantage over non-selective inhibition of IGF-1 R or its downstream PI3K/Akt pathway in terms of safety.

EGFR-T790M gatekeeper mutation is associated with 50% of cases of acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer.[4,8,9] Recently, mutant-selective EGFR-TKIs were developed, which inhibit EGFR with not only activating mutations, such as exon 19 in-frame deletion and L858R point mutation, but also T790M resistant mutation.[35] As the inhibitors were reported to have less activity for non-mutated EGFR, they may overcome T790M-mediated resistance and reduce adverse events, including skin toxicity. We found not only that Aki1 inhibition further augmented the efficacy of mutant EGFR-selective TKI and irreversible EGFR-TKI, but also that Aki1 constitutively associated with EGFR regardless of treatment with EGFR-TKI. In addition, Aki1 was detected in all tumors with acquired resistance, including tumors with *EGFR* T790M mutation. Our findings indicated the necessity of development of efficient Aki1 inhibitors, and suggested that combined use of Aki1 inhibitors may increase the therapeutic effects and may reduce adverse events concerning EGFR blockade of new generation EGFR-TKIs in *EGFR* mutant lung cancer.

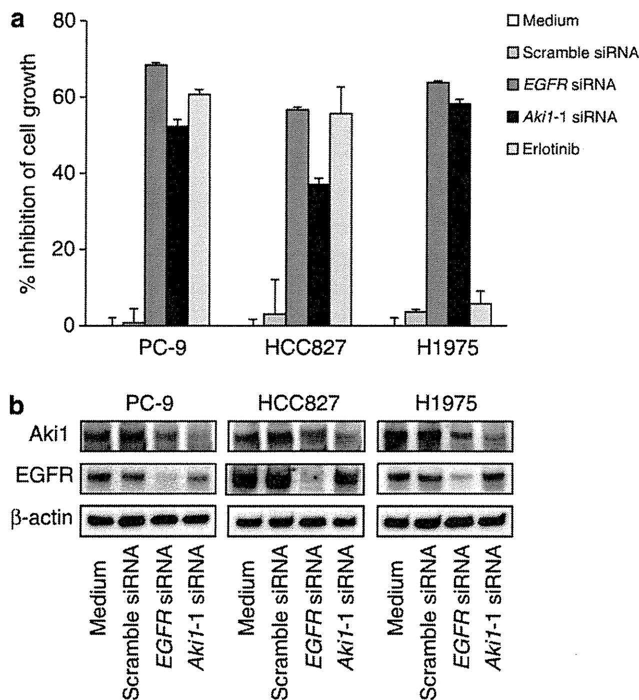

Figure 3. Comparison of efficacy between Aki1 knockdown and EGFR inhibition on cell viability. Cells were treated with *Aki1*-1 siRNA, *EGFR* siRNA, control scramble siRNA or erlotinib (1 μM). (**a**) After 72-h incubation, cell viability was determined by MTT assay. (**b**) After 24-h incubation, cells were lysed and the indicated proteins were detected by western blotting.

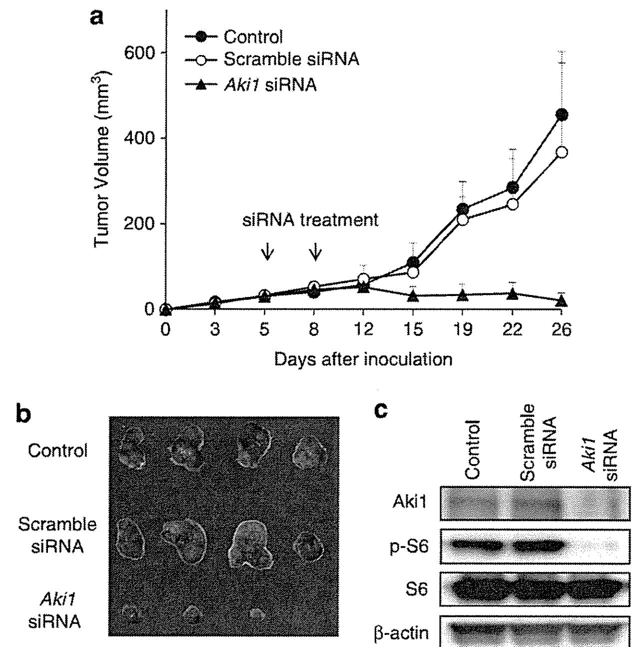

Figure 4. Therapeutic effects of *Aki1* knockdown against lung cancer cells with *EGFR* T790M secondary mutation *in vivo*. H1975 cells (5×10^6 cells per 100 μl of PBS) were injected subcutaneously into the flanks of 5-week-old male SCID mice. After cell inoculation, 50 μg of either scramble or *Aki1* siRNA complexed with invivofectamine was injected intratumorally on days 5 and 8. (**a**) Tumor size was measured twice a week and tumor volume was calculated as described in Materials and methods. (**b**) Macroscopic appearance of the tumors harvested on day 26. (**c**) The harvested tumors were examined for Aki1, and the inhibition of downstream signaling molecule, S6, in tumors by western blotting.

Figure 2. Effects of *Aki1* siRNA on cell viability and apoptosis in EGFR mutant human lung cancer cell lines. Cells were treated with *Aki1*-1 or control scramble siRNA. (**a**) After 72-h incubation, cell viability was determined by MTT assay. (**b**) After 24-h incubation with control scramble siRNA (lanes 1, 3 and 5) or *Aki1*-1 siRNA (lanes 2, 4 and 6), cells were lysed and the indicated proteins were detected by western blotting. (**c**) After 48-h incubation, cell apoptosis was determined with an Annexin V-FITC Apoptosis Detection Kit I. The numbers show percentages of early apoptotic cells.

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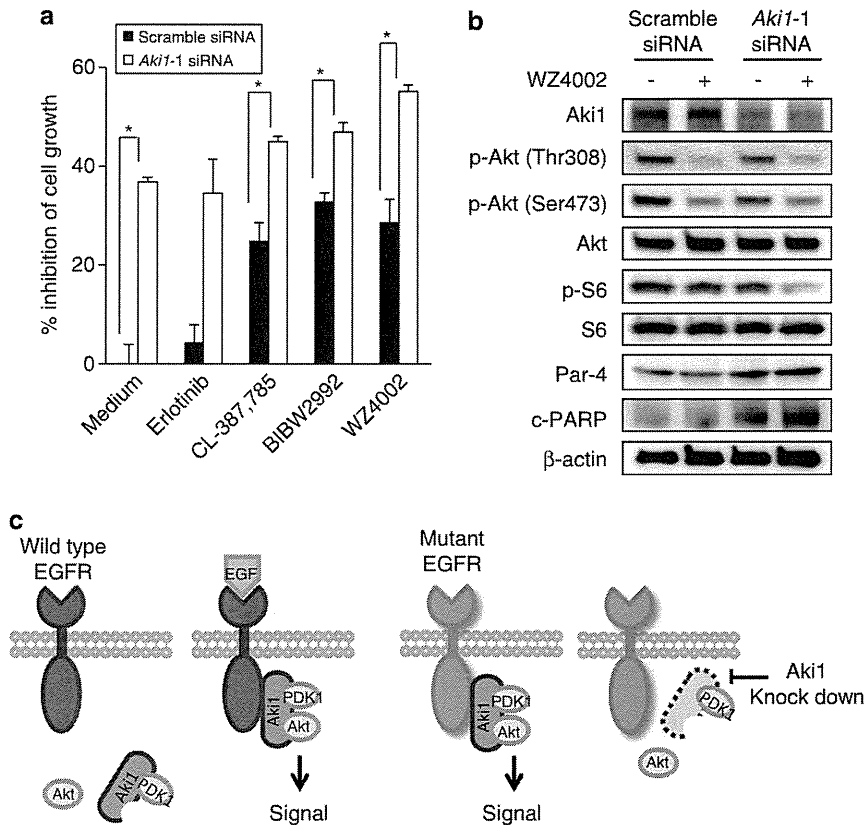

Figure 5. Effects of *Aki1* knockdown combined with new generation EGFR TKI in lung cancer with *EGFR* T790M secondary mutation. H1975 cells were treated with *Aki1*-1 or control scramble siRNA in the presence or absence of erlotinib (1 μM), CL-387, 785 (0.3 μM), BIBW2992 (0.1 μM) or WZ4002 (0.1 μM). (**a**) After 72-h incubation, cell viability was determined by MTT assay. *$P<0.01$, one-way ANOVA. (**b**) After 24-h incubation, cells were lysed and the indicated proteins were detected by western blotting. (**c**) Schema showing the role of Aki1 in cells with wild-type EGFR and mutant EGFR.

In conclusion, we demonstrated that Aki1 constitutively associates with EGFR activating mutation as well as T790M gatekeeper mutation has important roles as a determinant of receptor selective signaling for mutant EGFR, and mediates the survival signal to Akt. Our data provide a rationale for targeting Aki1 in *EGFR* mutant lung cancer patients, especially in cases with acquired resistance due to EGFR-T790M gatekeeper mutation. We are currently developing a drug delivery system for *Aki1* siRNA and small compounds with Aki1 inhibitory activity.

MATERIALS AND METHODS

Cell lines and reagents

The PC-9 and HCC827 human lung adenocarcinoma cell lines with EGFR-activating mutation (deletion in exon 19) were purchased from Immuno-Biological Laboratories (Gunma, Japan) and American Type Culture Collection (Manassas, VA, USA), respectively.[36] The H1975 human lung adenocarcinoma cell line with EGFR-L858R/T790M double mutation[10] was kindly provided by Dr John D Minna (University of Texas Southwestern Medical Center). The A549 human lung adenocarcinoma cell line, which expresses wild-type EGFR, was purchased from American Type Culture Collection. PC14PE6 human lung adenocarcinoma cell line, which expresses wild-type EGFR, was kindly provided by Dr Isaiah J Fidler (MD Anderson Cancer Center, Houston, TX, USA).[37] The human lung embryonic fibroblast MRC-5 (P30-35) and IMR-90 (P20-25) cell lines were obtained from RIKEN Cell Bank (Ibaraki, Japan). H1975, PC-9, HCC827, A549, and PC14PE6 cells were cultured in RPMI 1640, and MRC-5 and IMR-90 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (50 μg/ml), in a humidified CO_2 incubator at 37 °C. All experiments were performed in medium supplemented with 10% FBS. Erlotinib hydrochloride was obtained from Roche Pharma AG (Basel, Switzerland). CL-387, 785 was purchased from Calbiochem (San Diego, CA, USA). BIBW2992 and WZ4002 were purchased from Seleck Chemicals (Houston, TX, USA). Human wild-type *Aki1* cDNA in the pFLAG-CMV-2 vector was generated previously.[25] RNAi-resistant *Aki1* cDNA in the pFLAG-CMV-2 vector was generated by mutating CAAACTC of Aki1 siRNA-targeting sequence to TAAGTTA without changing the amino acid sequence.

Immunoprecipitation and western blotting

Tumor cells were incubated in 10 ml of RPMI 1640 with 10% FBS for 1 h. The cells were then washed twice with phosphate-buffered saline (PBS), harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenyl-methylsulfonyl fluoride) and flash-frozen on dry ice. After allowing the cells to thaw, the cell lysates were collected with a rubber scraper, sonicated and centrifuged at $14\,000 \times g$ (4 °C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots of 400 μg of total proteins were immunoprecipitated with the appropriate antibodies. In some experiments, tumor cells were incubated in 10 ml of RPMI 1640 with 0.1% FBS in the presence or absence of erlotinib (0.3 μM), CL-387, 785 (0.3 μM) for 48 h. In other experiments, tumor cells were incubated in 10 ml of RPMI 1640 with 0.1% FBS in the presence or absence of EGF (50 ng/ml) or IGF-1 (50 ng/ml) for 10 min. In some experiments, tumor cells were transfected with RNAi for 24 h incubation and then incubated in 10 ml of RPMI 1640 with 10% FBS in the presence or absence of WZ4002 (0.1 μM) for 1 h. Immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories, San Francisco, CA, USA). For western blotting assay, immunoprecipitates or cell lysates were subjected to SDS–PAGE (Bio-Rad, Hercules, CA, USA) and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, and the blots were then incubated at 4 °C overnight with anti-phospho-EGFR (Y1068), anti-Akt

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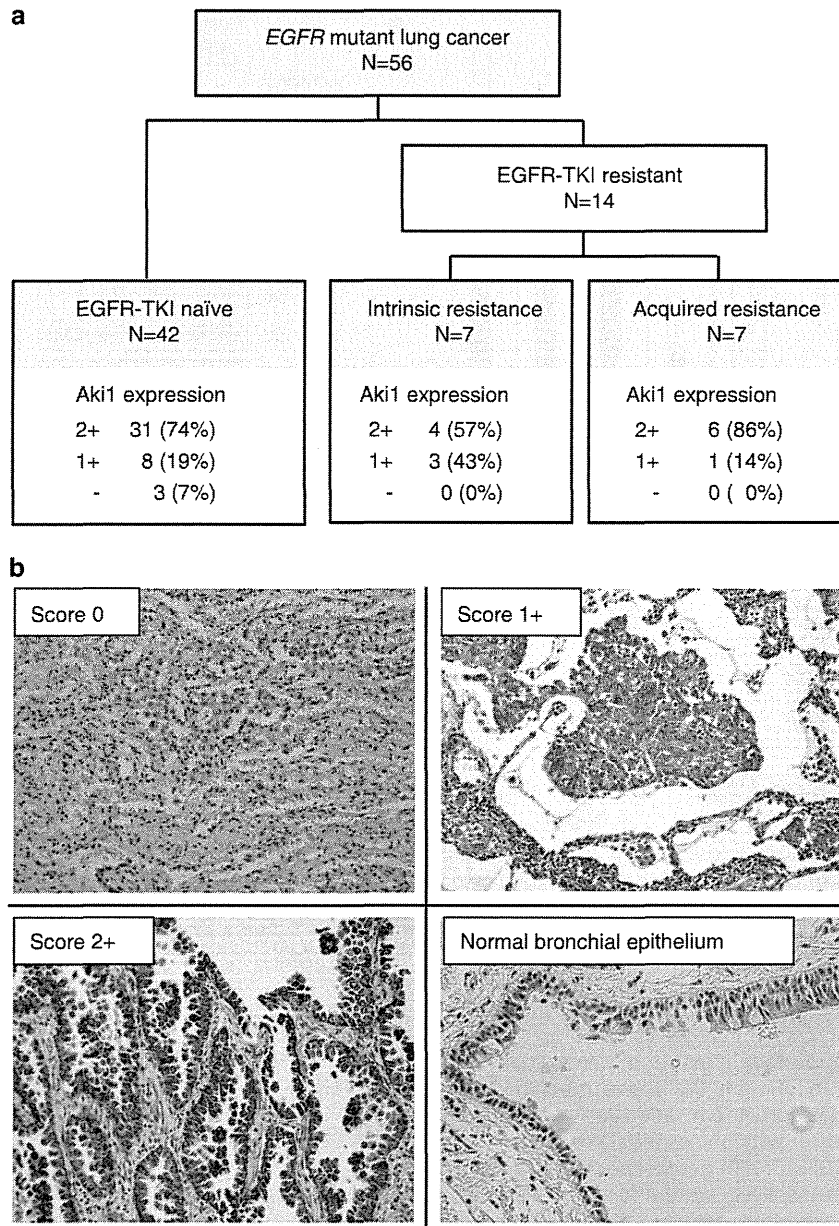

Figure 6. Aki1 is frequently expressed in *EGFR* mutant lung cancer. Clinical specimens from EGFR mutant lung cancer patients were stained for Aki1 by immunohistochemistry. (**a**) A total of 56 tumor specimens with EGFR-activating mutations were obtained from 56 lung adenocarcinoma patients. Of the 56 patients, 42 were EGFR-TKI naive, 7 tumors were from patients who showed intrinsic resistance to the EGFR-TKIs, gefitinib or erlotinib. Another seven tumors were from patients who showed acquired resistance to EGFR-TKIs. Of 42 EGFR-TKI naive tumors, the presence of Aki1 protein was scored as 2+ in 31 tumors (74%), 1+ in 8 tumors (19%) and – in 3 tumors (7%). Aki1 protein was detected diffusely in all of seven tumors with intrinsic resistance: 2+ in 4 (57%), 1+ in 3 (43%). Aki1 protein was detected diffusely in all of seven tumors with acquired resistance: 2+ in 6 (86%), 1+ in 1 (14%). (**b**) Representative staining results are shown.

(40D4), anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-Par-4, anti-cleaved PARP (Asp214), anti-phospho-S6 ribosomal protein (Ser235/236), anti-S6 ribosomal protein (5G10), anti-phospho-IGF-1 R (Tyr1131), DYKDDDDK (FLAG) tag antibody or anti-β-actin (13E5) antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-Aki1 (1:1000 dilution; Bethyl Laboratories, Montgomery, TX, USA), anti-PDK1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-human EGFR (1 μg/ml) or anti-human IGF-1 R (0.1 μg/ml) antibody (R&D Systems, Minneapolis, MN, USA). After washing three times, the membranes were incubated for 1 h at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

RNAi and proliferation assay *in vitro*

Duplexed Stealth RNAi (Invitrogen) against *Aki1*, *EGFR* and Stealth RNAi Negative Control Low GC Duplex no. 3 (Invitrogen, Carlsbad, CA, USA) were used for RNAi assay. Briefly, aliquots of 1×10^5 cells in 2 ml of antibiotic-free medium were plated on six-well plates and incubated at 37 °C for 24 h. The cells were then transfected with siRNA (250 pmol) or scramble RNA using Lipofectamine 2000 (5 μl) in accordance with the manufacturer's instructions (Invitrogen). After 24 h, the cells were washed twice with PBS. These partial cells were then used for western blotting and cell apoptosis assay. For proliferation assay, the cells were reseeded at 2×10^3 per well in 96-well plates, and incubated in antibiotic-containing RPMI 1640 with 10% FBS for 48 h. Otherwise, after 24 h of incubation, erlotinib (1 μM), CL-387, 785 (0.3 and 1 μM), BIBW2992 (0.1 and 0.3 μM) or WZ4002 (0.1, 0.3 μM) was added to each well, and incubation was continued for a further 48 h. These

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cells were then used for proliferation assay, which was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) dye reduction method. An aliquot of MTT solution (2 mg/ml; Sigma, St Louis, MO, USA) was added to each well followed by incubation for 2 h at 37 °C. The media were removed and the dark blue crystals in each well were dissolved in 100 μl of dimethyl sulfoxide (DMSO). Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 nm and 630 nm, respectively. The percentage of growth is shown relative to untreated controls.

Aki1 and *EGFR* knockdown were confirmed by western blotting analysis. The target sequences of siRNAs were as follows: *Aki1*-1, 5′-AGGAGCAGTT CAAACTCTGCATCAA-3′ (corresponding to nucleotides 2125–2168); *Aki1*-2, 5′-AACAAAGACAUCCAGAUCGCCAGGG-3′; *EGFR*, 5′-CGGAATAGGTATTGGT GAATTTAAA-3′ (corresponding to nucleotides 1014–1038). Each experiment was performed at least in triplicate, and three times independently.

Cell apoptosis assay

Cell apoptosis induced by *Aki1*-1 siRNA, *Aki1*-2 siRNA or Scramble siRNA was detected with an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen, Heidelberg, Germany) in accordance with the manufacturer's protocols as we described previously.[36] The analysis was performed on a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Xenograft studies in SCID mice and *in vivo* RNAi

Suspensions of H1975 cells (5×10^6 cells per 100 μl of PBS) were injected subcutaneously into the flanks of 5-week-old male SCID mice (Nihon Clea Co., Ltd, Tokyo, Japan). Tumor size was measured using digital calipers and tumor volume was calculated as $0.5 \times \text{length} \times (\text{width})^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

After cell inoculation, 50 μg of either scramble or Aki1 siRNA complexed with Invivofectamine (Invitrogen) was injected intratumorally on days 5 and 8. Tumors were harvested on day 26. siRNA and Invivofectamine complex was prepared in accordance with the manufacturer's instructions (Invitrogen). Aki1 knockdown in tumor tissue was confirmed by western blotting analysis.

Patients

A total of 56 tumor specimens with EGFR-activating mutations were obtained from 56 lung adenocarcinoma patients with written informed consent at the Kanazawa University Hospital (Kanazawa, Japan), Aichi Cancer Center Hospital (Nagoya, Japan), Osaka Medical Center (Osaka, Japan) and National Cancer Center Hospital East (Chiba, Japan) in studies with Institutional Review Board approval. Of the 56 patients, 42 were EGFR-TKI naive, seven showed intrinsic resistance, and the remaining seven patients showed partial response to initial EGFR-TKI treatment. As intrinsic resistance is not yet clearly defined, in the present study, we defined intrinsic resistant tumors as follows: response to treatment with an EGFR-TKI as defined by either documented stable disease or progressive disease (RECIST). Data for specimens from the seven patients who showed intrinsic resistance were obtained before EGFR-TKI treatment. For the seven patients who showed acquired resistance, tumor specimens were available after the development of acquired resistance to EGFR-TKI. Tumors with acquired resistance were defined as described previously.[38] Four of seven tumors from seven patients who showed acquired resistance had T790M secondary mutation.

Histology and immunohistochemistry

Immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded tissue sections of lung adenocarcinoma specimens. Sections 4-μm thick were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. After blocking the endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 12 min, the sections were treated with 5% normal horse serum. The sections were then reacted with primary antibody (1:100 dilution, rabbit polyclonal anti-CC2D1A antibody; Sigma-Aldrich Corp., St Louis, MO, USA) at 4 °C overnight. After washing with PBS, the sections were treated with biotin-conjugated anti-rabbit IgG (1:200 dilution) for 30 min at room temperature and allowed to react for 30 min with avidin–biotin–peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The DAB (3,3′-diaminobenzidine tetrahydrochloride) Liquid System (DakoCytomation, Glostrup, Denmark) was used to detect immunostaining. Omission of primary antibodies served as negative control.

Evaluation of immunohistochemical results

Aki1 immunoreactivity was evaluated as the percentage of cancer cells with positive cytoplasmic staining (0, <5%; 1+, 5%–50%; 2+, >50%). Positive cells were defined as those with staining intensity that was the same or greater than that of normal bronchial epithelium (Figure 6b). Evaluation was performed independently by two investigators (TY, HU) who were blind to individual clinical information about specimens.

Statistical analysis

The statistical significance of differences was analyzed by one-way ANOVA performed with GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

CONFLICT OF INTEREST

Seiji Yano received honoraria from Chugai Pharmaceutical Co., Ltd. and AstraZeneca. Seiji Yano received research funding from Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd. and Eisai Co., Ltd.

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Priority Report

Cancer Research

EGFR-TKI Resistance Due to *BIM* Polymorphism Can Be Circumvented in Combination with HDAC Inhibition

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Abstract

BIM (BCL2L11) is a BH3-only proapoptotic member of the Bcl-2 protein family. BIM upregulation is required for apoptosis induction by EGF receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKI) in *EGFR*-mutant forms of non–small cell lung cancer (NSCLC). Notably, a *BIM* deletion polymorphism occurs naturally in 12.9% of East Asian individuals, impairing the generation of the proapoptotic isoform required for the EGFR-TKIs gefitinib and erlotinib and therefore conferring an inherent drug-resistant phenotype. Indeed, patients with NSCLC, who harbored this host *BIM* polymorphism, exhibited significantly inferior responses to EGFR-TKI treatment than individuals lacking this polymorphism. In an attempt to correct this response defect in the resistant group, we investigated whether the histone deacetylase (HDAC) inhibitor vorinostat could circumvent EGFR-TKI resistance in *EGFR*-mutant NSCLC cell lines that also harbored the *BIM* polymorphism. Consistent with our clinical observations, we found that such cells were much less sensitive to gefitinib-induced apoptosis than *EGFR*-mutant cells, which did not harbor the polymorphism. Notably, vorinostat increased expression in a dose-dependent manner of the proapoptotic BH3 domain-containing isoform of BIM, which was sufficient to restore gefitinib death sensitivity in the *EGFR* mutant, EGFR-TKI–resistant cells. In xenograft models, while gefitinib induced marked regression via apoptosis of tumors without the *BIM* polymorphism, its combination with vorinostat was needed to induce marked regression of tumors with the *BIM* polymorphism in the same manner. Together, our results show how HDAC inhibition can epigenetically restore BIM function and death sensitivity of EGFR-TKI in cases of *EGFR*-mutant NSCLC where resistance to EGFR-TKI is associated with a common *BIM* polymorphism. *Cancer Res; 73(8); 2428–34. ©2013 AACR.*

Introduction

The EGF receptor (EGFR) tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, have shown marked therapeutic effects against non–small cell lung cancer (NSCLC) with *EGFR*-activating mutations, such as exon 19 deletions and L858R point mutations (1). About 20% to 30% of patients, however, show intrinsic resistance to EGFR-TKIs despite having tumors harboring these *EGFR* mutations. In addition, patients who respond initially later develop acquired resistance to EGFR-TKIs after varying periods of time (2). Among the molecular mechanisms associated with acquired resistance to EGFR-TKIs are (i) gatekeeper mutations in *EGFR* (i.e., a T790M second mutation), (ii) activation of bypass signaling caused by *Met* amplification or hepatocyte growth factor overexpression, (iii) transformation to small-cell lung cancer, and (iv) epithelial-to-mesenchymal transition (3, 4). Several therapeutic strategies, including new generation EGFR-TKIs and the combination of an EGFR-TKI and a Met-TKI, have been evaluated clinically in patients with *EGFR*-mutant NSCLC who acquired resistance to EGFR-TKIs (2). The mechanisms of intrinsic resistance, however, remain poorly understood.

Recently, a *BIM* deletion polymorphism was reported to be a novel mechanism of intrinsic resistance to EGFR-TKIs (5). BIM, also called BCL2L11, is a proapoptotic protein and a member of the Bcl-2 family. Gene products (such as BIM_{EL}, BIM_L, and BIM_S) with a BH3 domain, which is essential for apoptosis induction, antagonize antiapoptotic proteins (such as Bcl-2, Bcl-X_L, and Mcl-1) and activate proapoptotic proteins (such as BAX and BAK), thereby inducing apoptosis (6, 7). Activation of BAX and BAK induce cytochrome *c* release into the cytoplasm and result in activation of the caspase cascade (8). BIM is pivotal in apoptosis induced by EGFR-TKIs in *EGFR*-mutant NSCLC cells (9). The expression and degradation of BIM is regulated mainly by the MEK-ERK pathway (10). The *BIM* deletion polymorphism is relatively common in East Asian populations (12.9%), with 0.5% of individuals being

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homozygous for this deletion. During the transcription of *BIM*, either exon 3 or exon 4, the latter of which encodes the BH3 domain, is spliced out due to the presence of a stop codon and a polyadenylation signal within exon 3 (11). The *BIM* deletion polymorphism involves the deletion of a 2903 bp fragment in intron 2 and results in the preferential splicing of exon 3 over exon 4, generating a *BIM* isoform that lacks the BH3 domain (5). A retrospective analysis in patients with *EGFR*-mutant NSCLC showed that progression-free survival (PFS) following EGFR-TKI treatment was significantly shorter in patients with the *BIM* polymorphism (6.6 months) than with wild-type *BIM* (11.9 months; ref.5). Another study in patients with *EGFR*-mutant NSCLC treated with EGFR-TKIs also reported that PFS was significantly shorter in patients with BIM-low (4.3 months) than BIM-high (11.3 months) expressing tumors (12), suggesting that reduced expression of BIM with a BH3 domain is associated with an unfavorable response to EGFR-TKIs. To date, however, no therapeutic strategy has yet been developed for patients with *EGFR*-mutant NSCLC with low BIM expression.

Histone deacetylase (HDAC) is an enzyme that regulates chromatin remodeling and is crucial in the epigenetic regulation of various genes (13). Many compounds targeting HDAC have been developed, including vorinostat, an HDAC inhibitor approved by the United States Food and Drug Administration (FDA) for the treatment of patients with cutaneous T-cell lymphoma (14). In mantle cell lymphoma (MCL) cell lines and in cells from patients with MCL, vorinostat induced histone hyperacetylation on promoter regions and consequent transcriptional activation of proapoptotic *BH3*-only genes, including BIM (15). Using *in vitro* and *in vivo* models, we assessed whether the combination of vorinostat and gefitinib restored the expression of BIM protein with a BH3 domain in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism and overcame EGFR-TKI resistance associated with this polymorphism.

Materials and Methods

Cell lines and reagents

The NSCLC cell lines, PC-9, HCC827, and HCC2279, all of which have *EGFR* mutations, were obtained from Immuno-Biological Laboratories Co., ltd., the American Type Culture Collection (ATCC), and Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX), respectively. PC-3 cells, established from a Japanese female patient with NSCLC and with an exon 19 deletion in *EGFR*, and differing from the prostate cancer cell line PC-3 (ATCC CRL1435), were purchased from Human Science Research Resource Bank (JCRB0077: http://cellbank.nibio.go.jp/~cellbank/cgi-bin/search_res_det.cgi?DB_NUM=1&ID=252 = 1&ID = 252). PC-3 and the other 3 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium, respectively, each supplemented with 10% FBS and antibiotics. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza). The cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation (Osaka, Japan) by short tandem repeat analysis. Vorinostat and gefitinib were obtained from Selleck Chemicals and AstraZeneca, respectively.

Genotype and expression analysis of *BIM*

Genomic DNA was extracted from cells using DNeasy Blood and Tissue Kits (Qiagen), according to the manufacturer's protocol. Total RNA was extracted from cells using RNeasy PLUS Mini kits (Qiagen). PCR methods were used to detect the *BIM* deletion polymorphism in the samples and the level of expression of *BIM* isoforms (5).

Cell apoptosis

Cells (3×10^3) were seeded into each well of 96-well, white-walled plates, incubated overnight, and treated with the indicated compounds or vehicle [dimethyl sulfoxide (DMSO)] for 48 hours. Cellular apoptosis was analyzed with Caspase-Glo 3/7 assay kits (Promega), which measure caspase-3/7 activity, and PE-Annexin V Apoptosis Detection Kits (BD Biosciences, in accordance with the manufacturers' directions.

Apoptotic cells in tumor xenografts were detected by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining, using the DeadEnd Fluorometric TUNEL system (Promega), according to the manufacturer's protocol.

RNA interference

Duplexed Stealth RNAi (Invitrogen) against *BIM* and Stealth RNAi-negative control low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assays as described (4). The siRNA target sequences were 5′-CAUGAGUUGUGACAAAUC-AACACAA-3′ and 5′-UUGUGUUGAUUUGUCACAACUCAUG-3′ for BIM #1, and 5′-UGAGUGUGACCGAGAAGGUAGACAA-3′ and 5′-UUGUCUACCUUCUCGGUCACACUCA-3′ for BIM #2.

Western blot analysis

Western blotting was conducted with antibodies against phospho-EGFR (Tyr1068), Akt, phospho-Akt (Ser473), cleaved PARP, cleaved caspase-3, histone H3, acetylated histone H3 (Lys27), BIM, and β-actin (Cell Signaling Technology); and against phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, and EGFR (R&D Systems). Blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies specific to mouse or rabbit immunoglobulin G, with signals detected by enhanced chemiluminescence (Pierce Biotechnology).

Subcutaneous xenograft models

Male BALB/cAJcl-nu/nu mice, ages 5 to 6 weeks, were obtained from CLEA Japan Inc and injected subcutaneously into their flanks with cultured tumor cells (5×10^6 cells/0.1 mL/mouse). When tumor volumes reached 100 to 200 mm^3, the mice were randomized and treated once daily with gefitinib and/or vorinostat. Each tumor was measured in 2 dimensions, and the volume was calculated using the formula: tumor volume (mm^3) = $1/2 \times$ length (mm) $\times$ width $(mm)^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval No. AP-081088).

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Statistical analysis

Between group differences were analyzed by one-way ANOVA. All statistical analyses were conducted using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc.), with $P < 0.05$ considered statistically significant.

Results

EGFR-mutant NSCLC cell lines harboring the *BIM* deletion polymorphism have low susceptibility to gefitinib-induced apoptosis

We first examined the *BIM* deletion polymorphism in *EGFR*-mutant NSCLC cell lines by PCR. PC-9 and HCC827 had wild-type alleles, with a PCR product 4.2 kb in size. Consistent with a previous report (5), HCC2279 cells were heterozygous for the *BIM* deletion polymorphism, with PCR products 4.2 kb (wild-type) and 1.3 kb (2.9 kb deletion polymorphism) in size. Among the 7 additional cell lines with *EGFR* mutations (Supplementary Table S1), PC-3 was heterozygous for the *BIM* deletion polymorphism (Fig. 1A). Western blot analyses reveal that the expression of the proapoptotic BIM protein was markedly lower in PC-3 and HCC2279 than in PC-9 and HCC827 cells. Analysis of *BIM* isoform transcripts showed that cells with the *BIM* polymorphism expressed more exon 3- than exon 4-containing transcripts (Supplementary Fig. S1A and S1B). Treatment with gefitinib enhanced BIM expression, caspase-3/7 activities, and apoptosis in PC-9 and HCC827 cells much more than in PC-3 and HCC2279 cells (Fig. 1B; Supplementary Fig. S1C, S1D, and S2). Moreover, gefitinib did not increase caspase-3/7 activity in PC-9 and HCC827 cells treated with *BIM* siRNA (Fig. 1C), indicating the crucial role of BIM in apoptosis induction in *EGFR*-mutant NSCLC cells treated with EGFR-TKI. These observations clearly showed that *EGFR*-mutant NSCLC cells with the *BIM* deletion polymorphism are much less sensitive to gefitinib, as shown by induction of apoptosis, than cells with wild-type *BIM*.

Vorinostat upregulates BIM and efficiently induces apoptosis when combined with gefitinib

Because HDAC inhibition modulates the expression of various genes, including proapoptotic molecules (13), we hypothesized that the HDAC inhibitor, vorinostat, may sensitize *EGFR*-mutant NSCLC cells with the *BIM* polymorphism to gefitinib. In *EGFR*-mutated NSCLC cell lines, including PC-3 and HCC2279 cells, vorinostat dose dependently increased the expression of acetylated histone H3 and BIM with the BH3 domain (Fig. 2A, Supplementary Fig. S3A). We further explored whether the addition of vorinostat to gefitinib induced apoptosis in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism (Fig. 2B and D). In HCC827 and PC-9 cells, which contain only wild-type *BIM*, gefitinib inhibited downstream signaling,

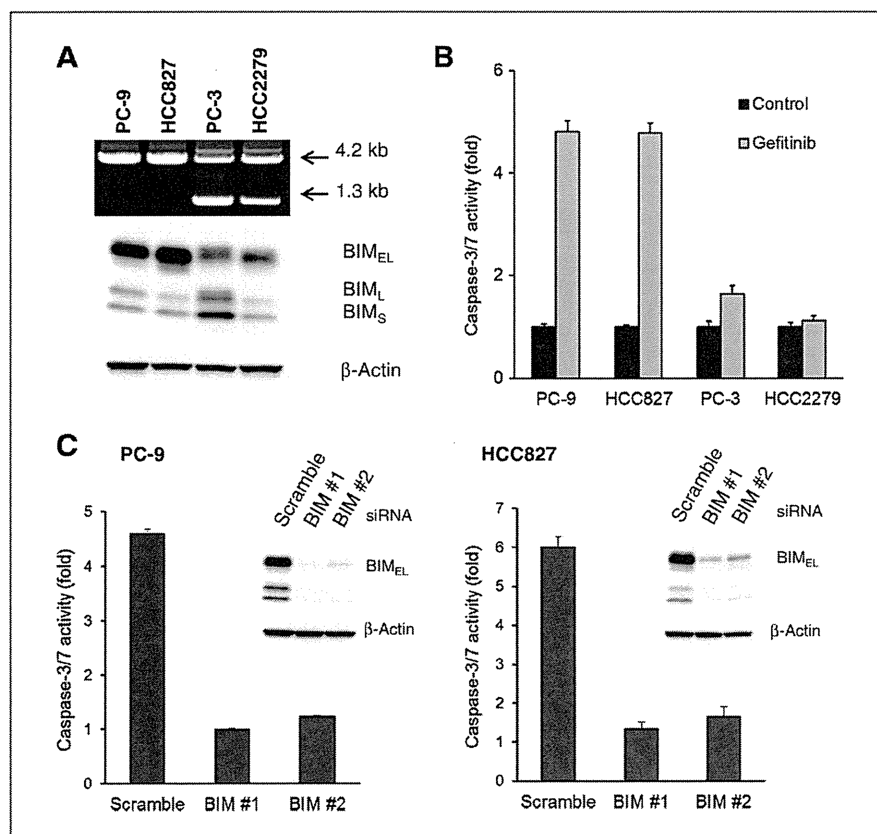

Figure 1. *EGFR*-mutated NSCLC cell lines harboring the *BIM* deletion polymorphism show low susceptibility to gefitinib-induced apoptosis. A, top, PCR products from the 4 *EGFR*-mutated NSCLC cell lines generated by primers flanking the deletion. PCR products 4.2 kb and 1.3 kb in size correspond to the alleles without and with the deletion, respectively, with the presence of both products indicating heterozygosity for the deletion polymorphism. Bottom, the levels of expression of the proteins BIM_{EL}, BIM_L, and BIM_S in each cell line. B, cell lines were treated with gefitinib (1 μmol/L) or DMSO control for 48 hours, and the activity of caspase-3/7 was measured using Caspase-Glo3/7 assay kits. Each bar represents the mean ± SD. C, PC-9 (left) and HCC827 (right) cells were transfected with *BIM* or control siRNA for 24 hours before gefitinib (1 μmol/L) treatment for 48 hours, and the activity of caspase-3/7 was measured as in B. Each bar indicates the mean ± SD. Lysates were collected and proteins were analyzed by Western blotting.

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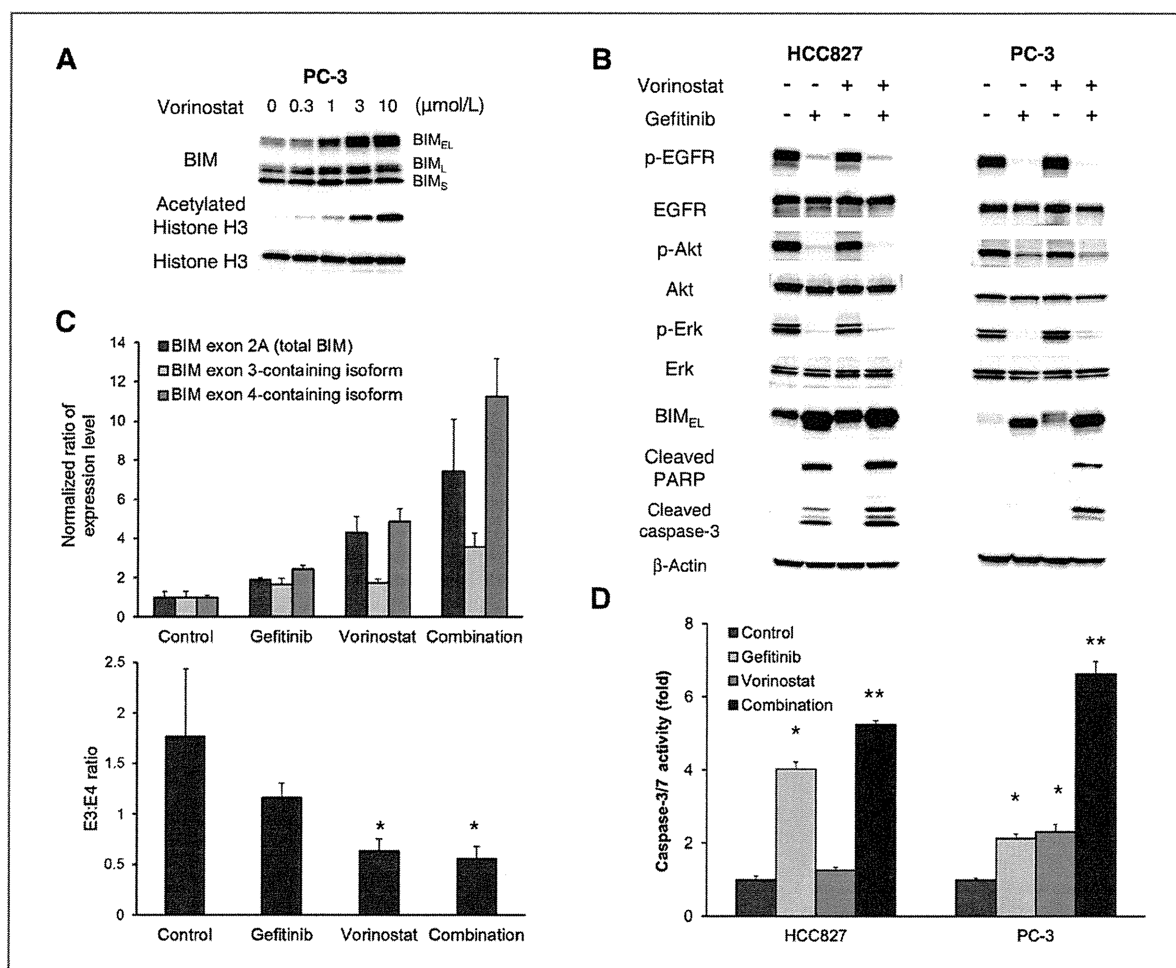

Figure 2. Upregulation of BIM by vorinostat enhances induction of apoptosis in *EGFR*-mutated NSCLC cell line with the *BIM* polymorphism. A, PC-3 cells were incubated with serial dilutions of vorinostat for 24 hours. The cell lysates were harvested and the indicated proteins were analyzed by Western blotting. B, HCC827 cells (left) and PC-3 cells (right) were incubated with gefitinib (1 μmol/L) and/or vorinostat (3 μmol/L) for 48 hours. The cell lysates were harvested and the indicated proteins were determined by Western blotting. C, PC-3 cells were treated with gefitinib (1 μmol/L) and/or vorinostat (3 μmol/L) for 12 hours. The amounts of the various transcripts containing exon 2A, 3, or 4 are expressed as normalized ratios relative to actin (top). Ratio of exon 3-containing transcripts to exon 4-containing transcripts in PC-3 cells after treatment with each compound. *, $P < 0.05$ versus control. Bar indicates the mean ± SD. D, apoptosis was analyzed by measurement of caspase-3/7 activity. *, $P < 0.05$ gefitinib or vorinostat versus control; **, $P < 0.05$ combination versus control and single agents. Bars represent the mean ± SD.

including the phosphorylation of EGFR, Erk, and Akt, resulting in apoptosis, as shown by the expression of cleaved PARP and cleaved caspase-3. The further addition of vorinostat augmented BIM expression and caspase-3/7 activity. In PC-3 and HCC2279 cells, which contain the *BIM* polymorphism, however, treatment with gefitinib alone induced minimal apoptosis, although the phosphorylation of EGFR, Erk, and Akt was inhibited, whereas the combination of vorinostat and gefitinib markedly increased the expression of BIM, as well as of cleaved PARP and cleaved caspase-3 (Fig. 2B and Supplementary Fig. S3B). This combination also augmented caspase-3/7 activity compared with that of gefitinib or vorinostat alone (Fig. 2D and Supplementary Fig. S3C), but this activation of caspase-3/7 was inhibited by knockdown of *BIM* (Supplementary Fig. S4A and S4B). Conversely, overexpression of BIM_{EL} itself stimulated caspase-3/7 activities in cells with the *BIM* polymorphism, with these activities further enhanced by gefitinib treatment (Supplementary Fig. S4C and S4D). These results indicate that BIM mediates the activation of caspase-3/7 induced by gefitinib and vorinostat. Analysis of *BIM* transcripts revealed that vorinostat alone induced *BIM* mRNA, which was enhanced by the inclusion of gefitinib. Moreover, vorinostat treatment preferentially induced transcripts containing exon 4 over those containing exon 3 (Fig. 2C). These results indicate that the combination of vorinostat and gefitinib inhibits HDAC and increases the expression of BIM protein with the BH3 domain, thereby sensitizing *EGFR*-mutant NSCLC cells with the *BIM* polymorphism to apoptosis *in vitro*.

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Combined treatment with vorinostat with gefitinib shrinks tumors produced by *EGFR*-mutant NSCLC cells with the *BIM* polymorphism

We next determined the *in vivo* efficacy of vorinostat and gefitinib. Gefitinib alone almost completely shrunk xenograft tumors induced by HCC827 cells (Fig. 3A). Although gefitinib monotherapy prevented the enlargement of tumors produced by PC-3 cells, which harbor the *BIM* polymorphism, it did not induce their complete regression, indicating that PC-3 cells remained less susceptible to gefitinib *in vivo*. Under these experimental conditions, vorinostat monotherapy inhibited tumor growth slightly, whereas the combination of vorinostat with gefitinib resulted in marked tumor shrinkage (Fig. 3B). None of the mice treated with these agents showed any macroscopic adverse effects, including loss of body weight (data not shown).

To clarify the mechanisms by which vorinostat and gefitinib act *in vivo*, we assessed tumor-cell apoptosis by TUNEL staining. Gefitinib treatment increased the number of apoptotic cells in HCC827 tumors but had little effect on PC-3 tumors (Fig. 4A and B), indicating that *EGFR*-mutant NSCLC cells with the *BIM* polymorphism are refractory to gefitinib-induced apoptosis *in vivo* as well as *in vitro*. Importantly, although vorinostat alone had little effect on apoptosis, the combination of vorinostat and gefitinib induced marked apoptosis in PC-3 tumors (Fig. 4A and B). Western blot analyses showed that gefitinib induced cleavage of caspase-3 in HCC827, but not in PC-3, tumors. In PC-3 tumors, treatment with gefitinib or vorinostat had little effect on caspase-3 cleavage, whereas their combination increased BIM expression and the cleavage of caspase-3 (Fig. 4C and D). These findings indicate that the combination of vorinostat and gefitinib increases BIM protein expression and induces tumor-cell apoptosis, thereby shrinking tumors produced by *EGFR*-mutant NSCLC cells with the *BIM* polymorphism.

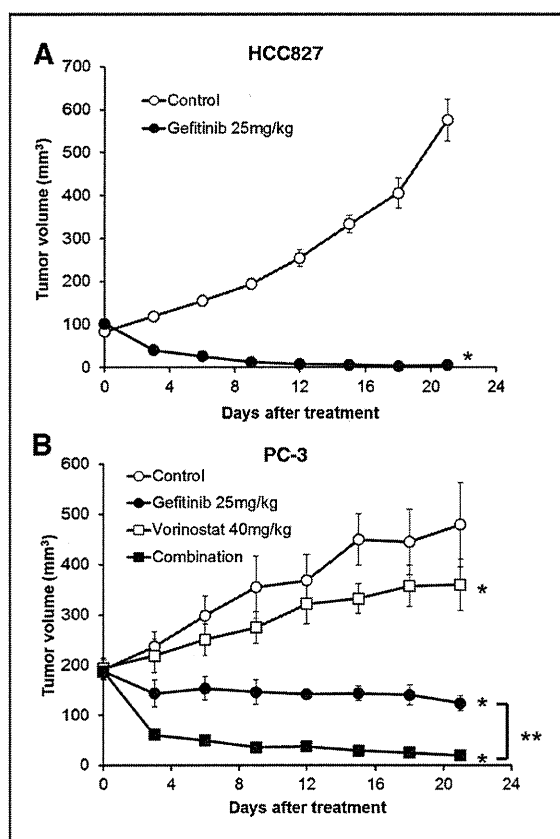

Figure 3. Antitumor activity of gefitinib and/or vorinostat in mouse xenograft models of HCC827 and PC-3 tumors. Nude mice bearing established tumors with HCC827 (A) or PC-3 (B) cells were treated with 25 mg/kg gefitinib and/or 40mg/kg vorinostat once daily for 21 days. Tumor volume was measured using calipers on the indicated days. Mean ± SE tumor volumes are shown for groups of 4 to 5 mice. *, $P < 0.05$ versus control, **, $P < 0.05$ versus gefitinib by one-way ANOVA.

Discussion

EGFR-mutant NSCLC cells with the *BIM* deletion polymorphism show impaired generation of BIM with the proapoptotic BH3 domain, as well as resistance to EGFR-TKI–induced apoptosis (5). We have shown here that treatment of cells with the combination of vorinostat, a HDAC inhibitor, and gefitinib, an EGFR-TKI, restored the expression of BIM protein with a BH3 domain (predominantly BIM_{EL}), induced apoptosis, and overcame gefitinib resistance *in vitro* and *in vivo*.

Although vorinostat preferentially induced expression of BIM containing the BH3 domain, its exact mechanisms of action remain unclear. The wild-type allele may be more susceptible to the effects of HDAC inhibition than the deletion allele due to differences in the acetylation status of these alleles. Alternatively, vorinostat may affect the splicing process, resulting in the production of exon 4- rather than exon 3-containing transcripts from the deletion polymorphism allele as HDAC has been found to affect the splicing of RNA (16).

Vorinostat has been shown to induce the expression of several genes other than *BIM* (13). However, we found that BIM was pivotal not only for gefitinib-induced apoptosis but also when combined with vorinostat. Moreover, the combination of vorinostat and gefitinib increased BIM expression and markedly induced apoptosis in PC-3 and HCC2279 cells. Collectively, these findings strongly suggest that vorinostat promotes gefitinib-induced apoptosis in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism, primarily by increasing BIM expression. Several other mechanisms, including inhibition of epigenetic modifications leading to a drug-tolerant state (17) and transition of cancer cells from a resistant mesenchymal state to an E-cadherin–expressing epithelial state (18) may be also involved.

Both the *BIM* polymorphism and *EGFR* mutations are more prevalent in East Asian than in Caucasian populations. Few East Asian patients with *EGFR*-mutant NSCLC show a complete response to EGFR-TKIs (1). This incomplete response, including intrinsic resistance, may be due, in part, to low BIM expression associated with the *BIM* polymorphism (6). Our preclinical data indicate that vorinostat increases BIM even in *BIM*-wild type *EGFR*--mutant NSCLC cells. However, a clinical trial with erlotinib and entinostat, an HDAC inhibitor, in

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