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

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

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Abstract

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-tyrosine kinase inhibitor (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/PDK1/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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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 to 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 tyrosine kinase inhibitor 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 ATP, 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 endpoints 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, whiles, 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 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)**. While 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 phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1(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 5 human lung adenocarcinoma cells with or without

EGFR mutations, comparing that in 2 human lung embryonic fibroblast cell lines, by Western blotting **(Fig. 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 **(Fig. 1B)**. Consistent with the results of previous studies **(25)**, Aki1 did not associate with IGF-1R, irrespective of IGF-1 stimulation, indicating selective binding of Aki1 to EGFR **(Fig. 1B)**. Moreover, treatment with EGFR-TKI didn't affect the association between Aki1 and

EGFR/PDK1/Akt **(Fig. 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, PC14PE6) **(Fig. 2A, 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 **(Fig. S3A)**. Treatment with *Aki1* siRNA attenuated Aki1 protein expression and cell viability in parental and even in wild-type *Aki1*-transfected cells **(Fig. S3B,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 **(Fig. 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 **(Fig. 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 **(Fig. S5A, S5B)**. 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 **(Fig. 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 **(Fig. 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, while erlotinib had no effect **(Fig. 3A,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 **(Fig. 4A, B)**. We confirmed knockdown of Aki1 and the inhibition of downstream signaling molecule, S6, in tumors by Western blotting **(Fig. 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, while 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 **(Fig. 5A, Fig. S4)**. Consistent with these findings, *Aki1* knockdown decreased the levels of Akt and S6 phosphorylation, and increased the level of Par-4 and cleaved PARP, when combined with WZ4002 **(Fig. 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 **(Fig. 6A, 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 **(Fig. S6)**. Forty-two tumors were obtained from EGFR-TKI naïve patients. Seven tumors were

from patients who showed intrinsic resistance to the EGFR-TKIs, gefitinib or erlotinib. Another 7 tumors were from patients who showed acquired resistance to EGFR-TKIs **(Fig. 6A)**. Of 42 EGFR-TKI naïve 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 7 tumors with intrinsic resistance: 2+ in 4 (57%), 1+ in 3 (43%). Aki1 protein was detected diffusely in all of 7 tumors with acquired resistance: 2+ in 6 (86%), 1+ in 1 (14%) **(Fig. 6A)**. Aki1 was detected in all tumors with acquired resistance, including 4 tumors with *EGFR* T790M mutation **(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 present of EGFR-TKI. Silencing of *Aki1* induces apoptosis of *EGFR* mutant lung cancer cells, indicating that Aki1 plays 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 **(Fig. 1B)**. 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-1R 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-1R 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-1R antibody. A preliminary report of toxicity from a phase II trial with the anti-IGF-1R antibody demonstrated severe adverse events, including hyperglycemia **(34)**. These findings indicated difficulty of targeting IGF-1R in cancer. As Aki1 is an EGFR-selective scaffold protein, Aki1 inhibition may have advantage over nonselective inhibition of IGF-1R 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.

In conclusion, we demonstrated that Aki1 constitutively associates with EGFR with activating mutation as well as T790M gatekeeper mutation, plays 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), 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 (M.D. Anderson Cancer Center, Houston, TX) **(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, and HCC827 cells were cultured in RPMI 1640, and MRC-5 and IMR-90 cells were cultured in DMEM 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). BIBW2992 and WZ4002 were purchased from Seleck Chemicals (Houston, TX). 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 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 phenylmethylsulfonyl 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 14000 × *g* (4°C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). 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). For Western blotting assay, immunoprecipitates or cell lysates were subjected to SDS-PAGE (Bio-Rad, Hercules, CA) 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 (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-1R (Tyr1131), DYKDDDDK (FLAG) tag antibody, or anti-β-actin (13E5) antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA), anti-Aki1 (1:1000 dilution; Bethyl Laboratories, Montgomery, TX), anti-PDK1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-human EGFR (1 μg/mL) or anti-human IGF-1R (0.1 μg/mL) antibody (R&D Systems, Minneapolis, MN). 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 #3 (Invitrogen) were used for RNAi assay. Briefly, aliquots of 1×10^5 cells in 2 mL of antibiotic-free medium were plated on 6-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, 1 μM), BIBW2992 (0.1, 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 cells were then used for proliferation assay, which was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method. An aliquot of MTT solution (2 mg/mL; Sigma, St. Louis, MO) 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 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'-AGGAGCAGTTCAAACTCTGCATCAA-3' (corresponding to nucleotides 2125 – 2168); *Aki1*-2, 5'-AACAAAGACAUCCAGAUCGCCAGGG-3'; *EGFR*, 5'-CGGAATAGGTATTGGTGAATTTAAA-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).

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). 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 naïve, 7 showed intrinsic resistance, and the remaining 7 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 7 patients who

showed intrinsic resistance were obtained before EGFR-TKI treatment. For the 7 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 7 tumors from 7 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, MO) 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). 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 **(Fig. 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). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Conflicts of Interest

Seiji Yano received honoraria from Chugai Pharmaceutical Co., Ltd. and AstraZeneca.

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Figure legends

Figure1

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.

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, 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.

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 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.

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 naïve, 7 tumors were from patients who showed intrinsic resistance to the EGFR-TKIs, gefitinib or erlotinib. Another 7 tumors were from patients who showed acquired resistance to EGFR-TKIs. Of 42 EGFR-TKI naïve 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 7 tumors with intrinsic resistance: 2+ in 4 (57%), 1+ in 3 (43%). Aki1 protein was detected diffusely in all of 7 tumors with acquired resistance: 2+ in 6 (86%), 1+ in 1 (14%). **(B)** Representative staining results are shown.

Fig. 1

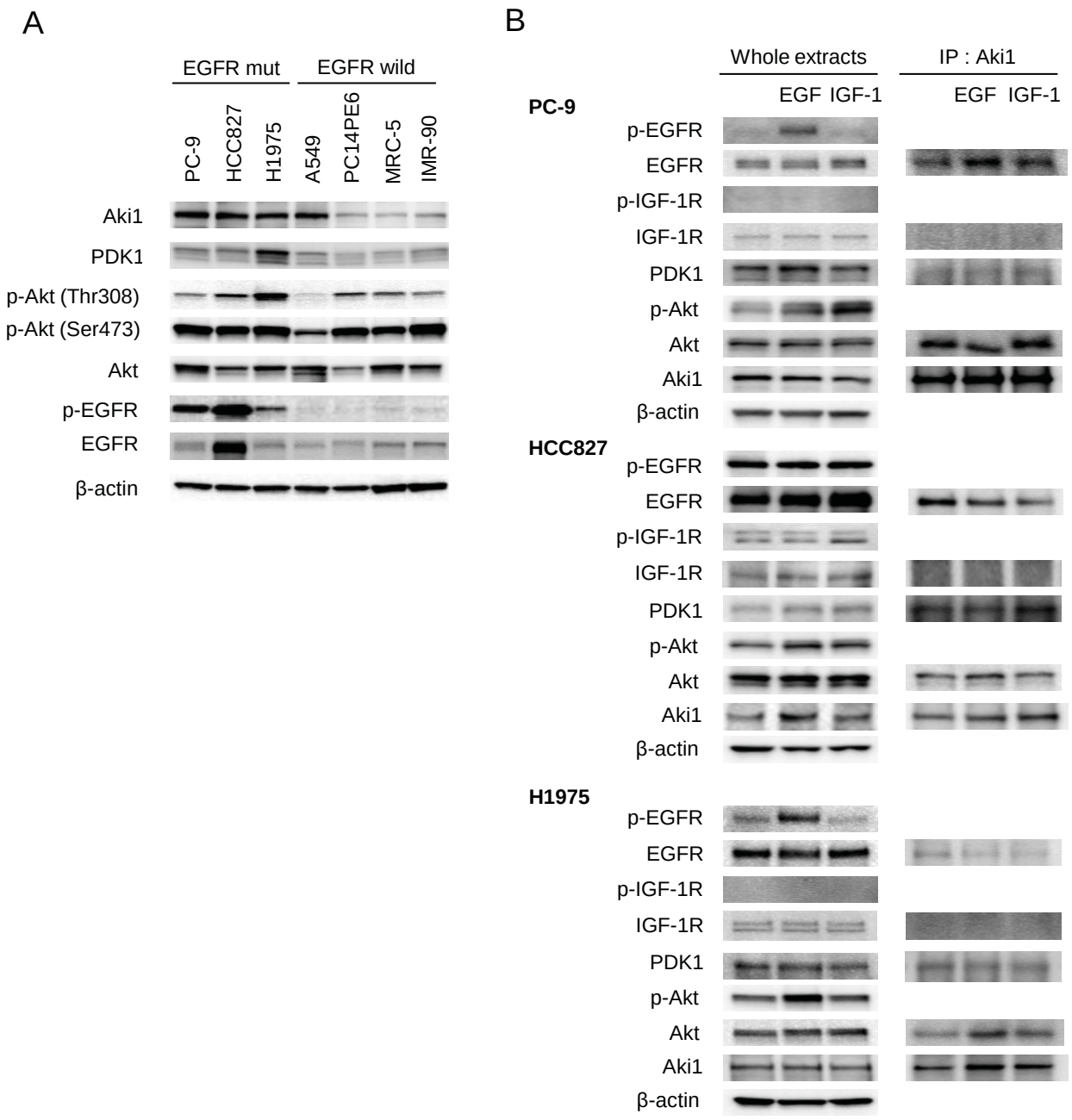
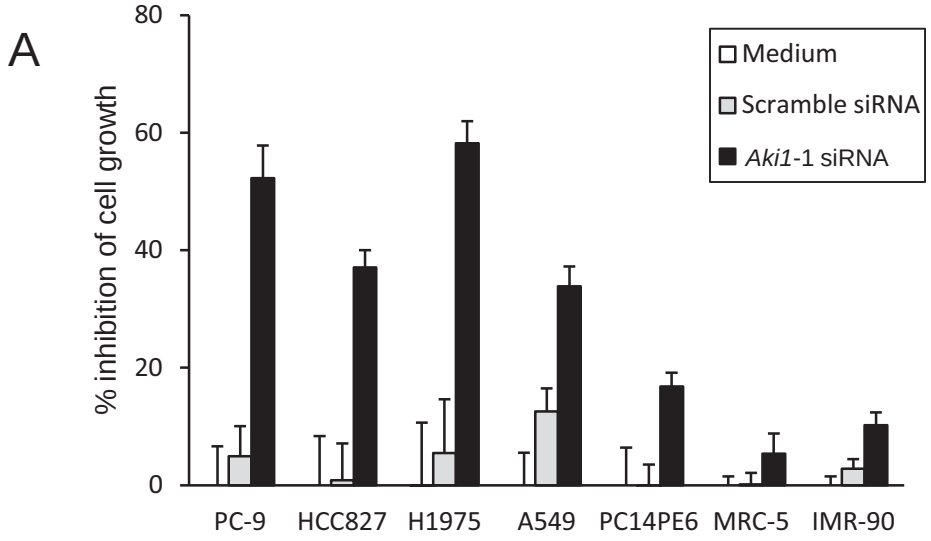
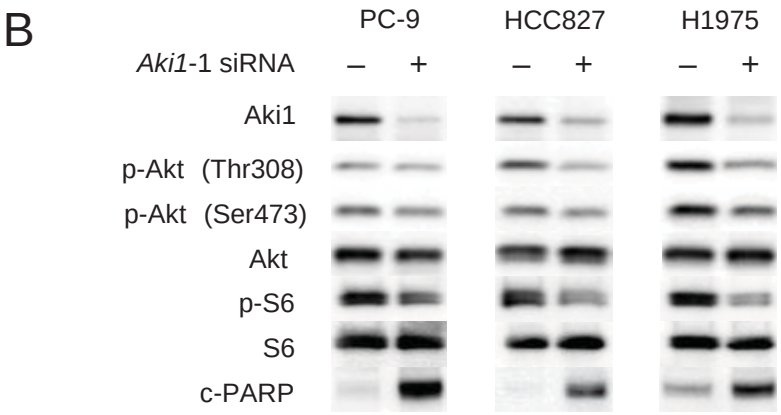

A
EGFR mut
EGFR wild
PC-9
HCC827
H1975
A549
PC14PE6
MRC-5
IMR-90
Aki1
PDK1
p-Akt (Thr308)
p-Akt (Ser473)
Akt
p-EGFR
EGFR
β-actin
B
Whole extracts
IP : Aki1
EGF
IGF-1
PC-9
HCC827
H1975
p-EGFR
EGFR
p-IGF-1R
IGF-1R
PDK1
p-Akt
Akt
Aki1
β-actin

Fig. 2


A
80
60
40
20
0
% inhibition of cell growth
Medium
Scramble siRNA
Aki1-1 siRNA
PC-9
HCC827
H1975
A549
PC14PE6
MRC-5
IMR-90


B
PC-9
HCC827
H1975
Aki1-1 siRNA
– + – + – +
Aki1
p-Akt (Thr308)
p-Akt (Ser473)
Akt
p-S6
S6
c-PARP

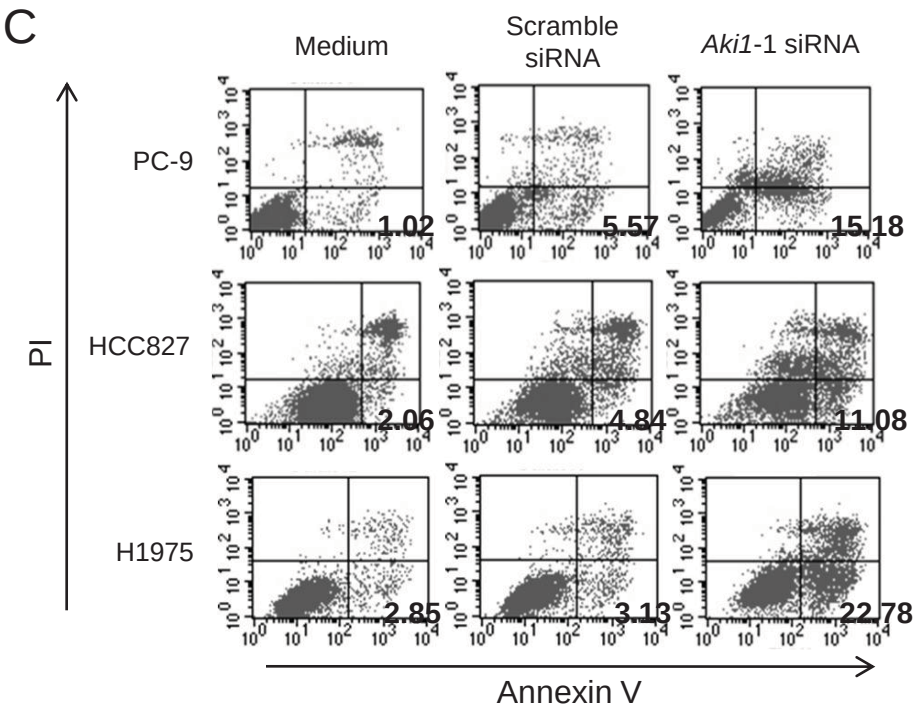
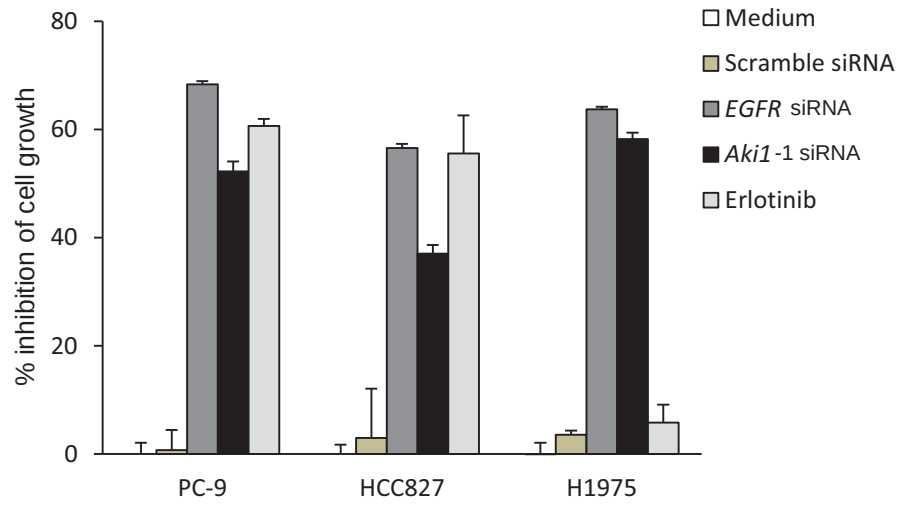

C
Medium
Scramble siRNA
Aki1-1 siRNA
PC-9
HCC827
H1975
PI
Annexin V
1.02
5.57
15.18
2.06
4.84
11.08
2.85
3.13
22.78

Fig. 3

A


80
60
40
20
0
% inhibition of cell growth
PC-9
HCC827
H1975
Medium
Scramble siRNA
EGFR siRNA
Aki1-1 siRNA
Erlotinib

B

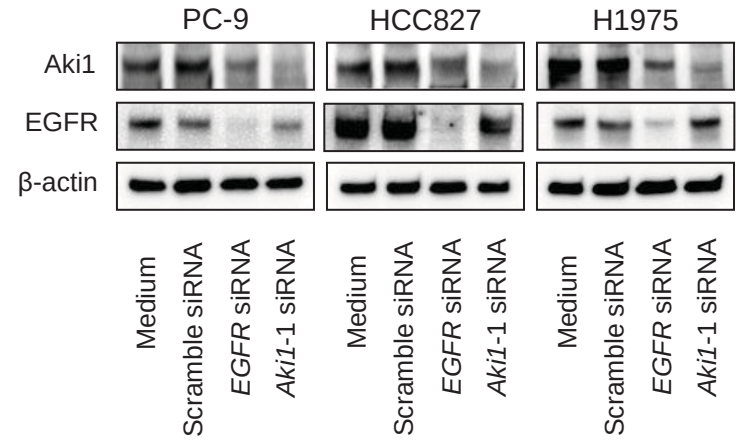

PC-9
HCC827
H1975
Aki1
EGFR
β-actin
Medium
Scramble siRNA
EGFR siRNA
Aki1-1 siRNA
Medium
Scramble siRNA
EGFR siRNA
Aki1-1 siRNA
Medium
Scramble siRNA
EGFR siRNA
Aki1-1 siRNA

Fig. 4

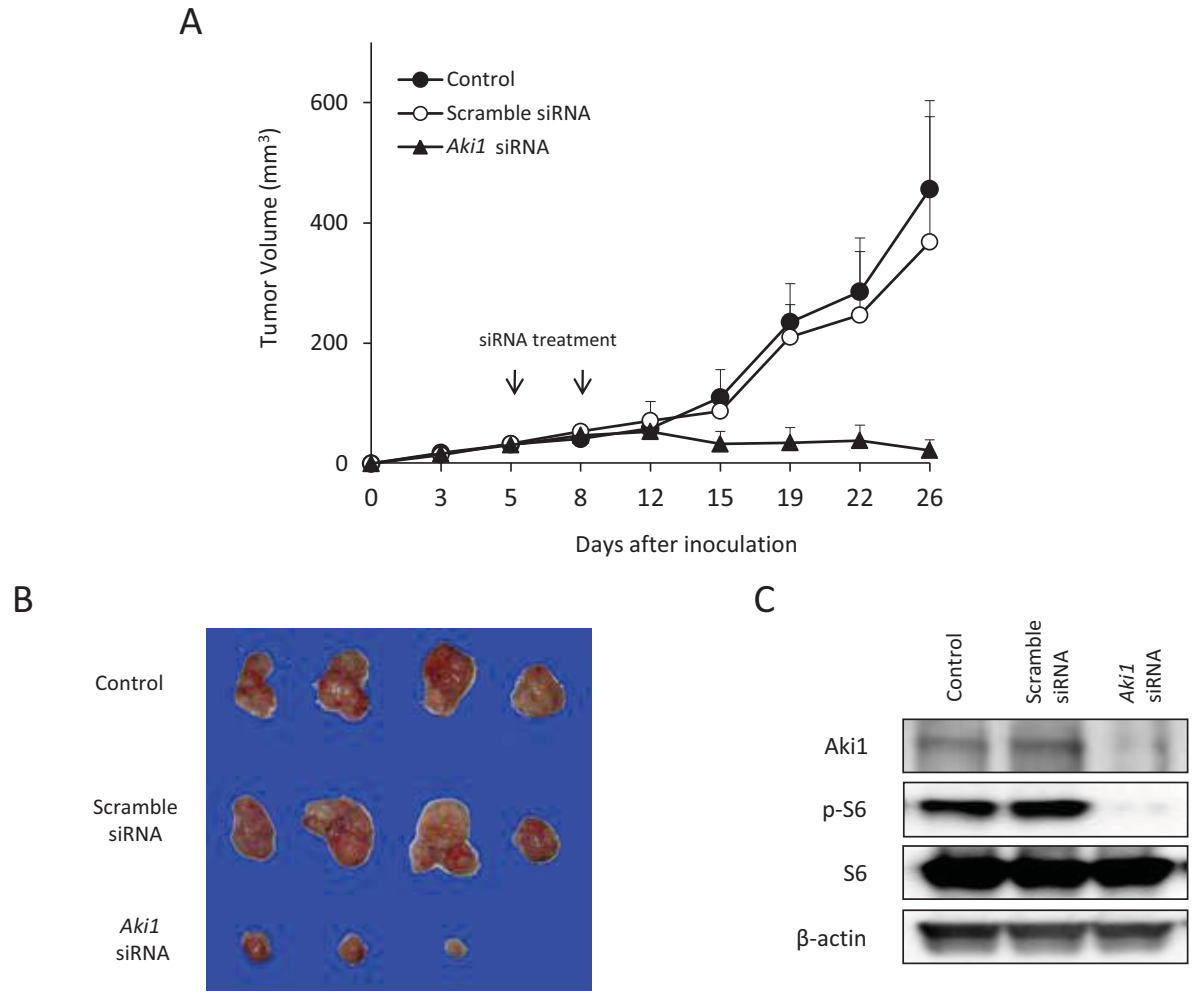

A
Control
Scramble siRNA
Aki1 siRNA
Tumor Volume (mm3)
600
400
200
0
siRNA treatment
0
3
5
8
12
15
19
22
26
Days after inoculation
B
Control
Scramble siRNA
Aki1 siRNA
C
Control
Scramble siRNA
Aki1 siRNA
Aki1
p-S6
S6
β-actin

Fig. 5

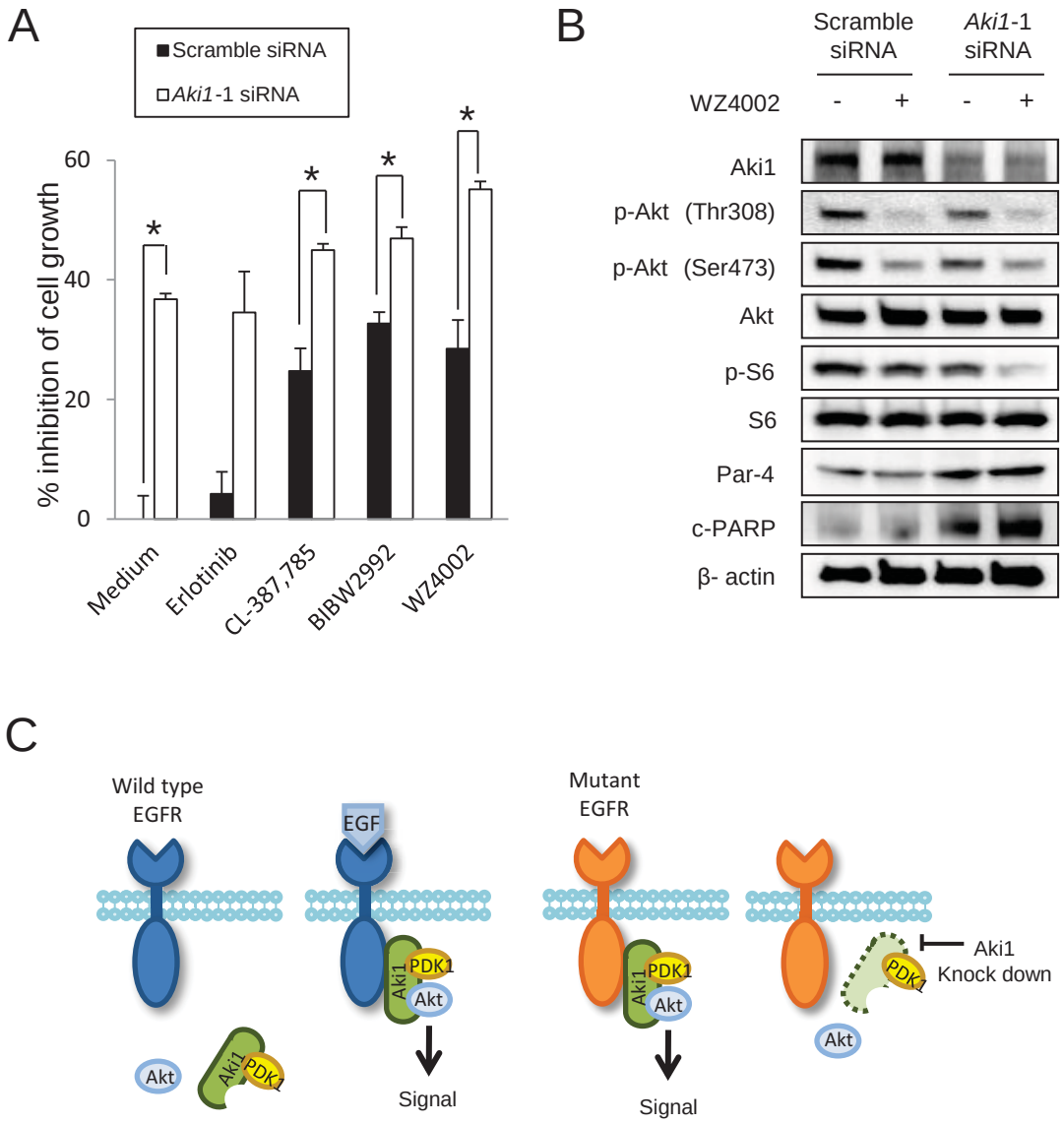
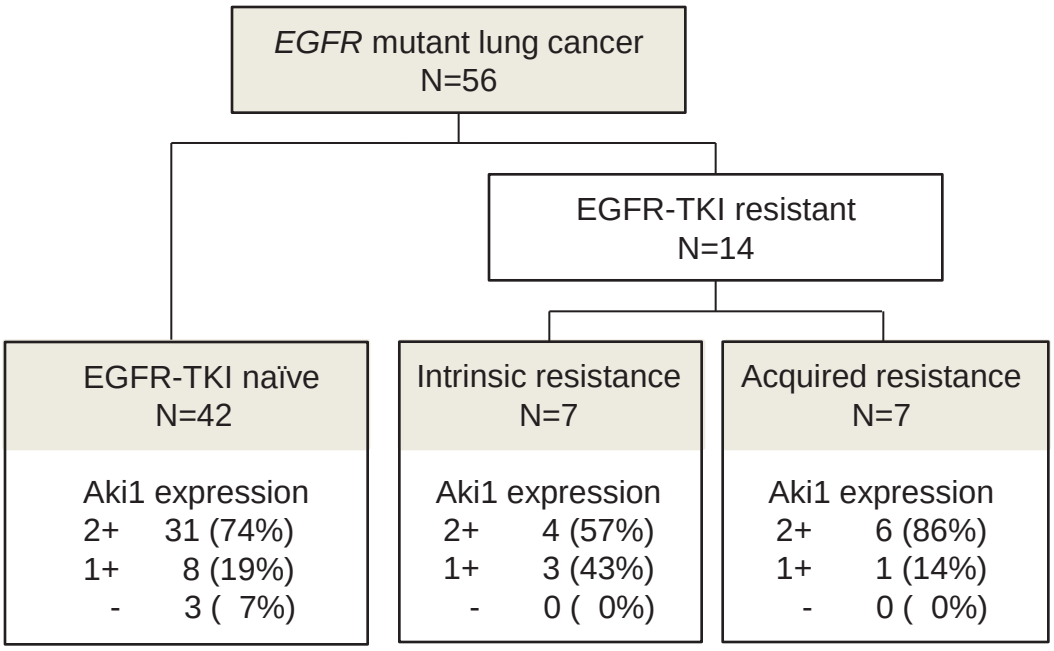
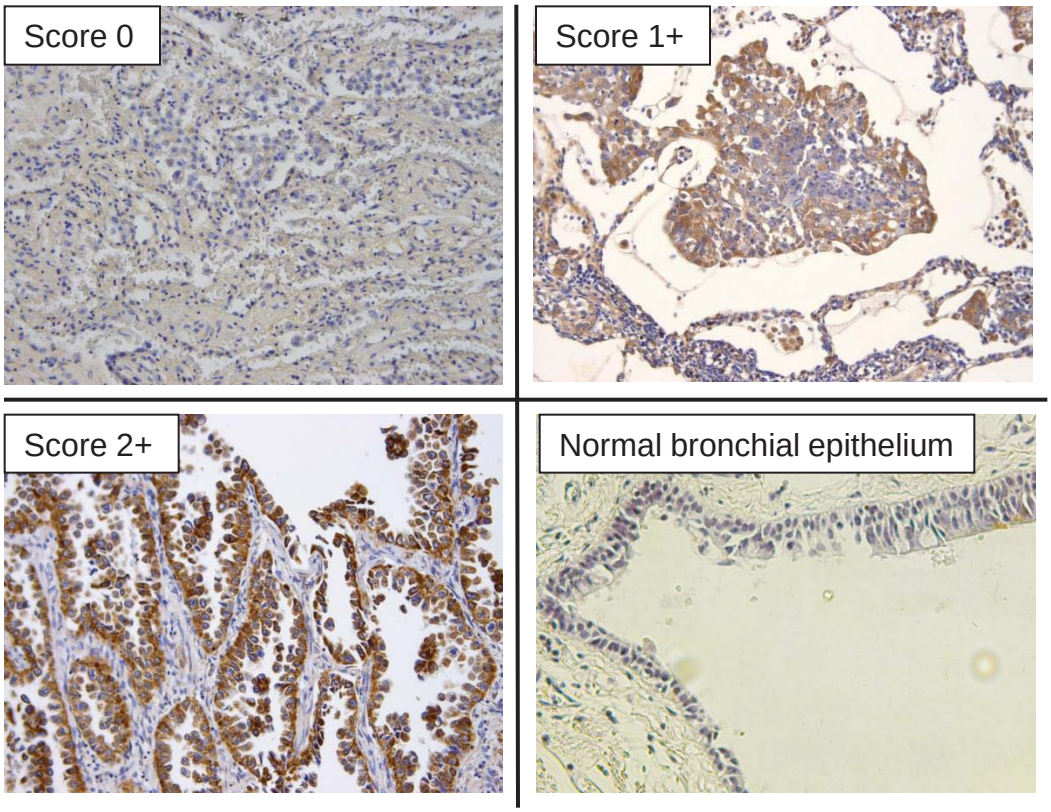

A
Scramble siRNA
Aki1-1 siRNA
% inhibition of cell growth
60
40
20
0
*
Medium
Erlotinib
CL-387,785
BIBW2992
WZ4002
B
Scramble siRNA
Aki1-1 siRNA
WZ4002 - + - +
Aki1
p-Akt (Thr308)
p-Akt (Ser473)
Akt
p-S6
S6
Par-4
c-PARP
β- actin
C
Wild type EGFR
EGF
Akt
Aki1
PDK1
Signal
Mutant EGFR
Aki1 Knock down

Fig. 6

A


EGFR mutant lung cancer
N=56
EGFR-TKI resistant
N=14
EGFR-TKI naïve
N=42
Aki1 expression
2+ 31 (74%)
1+ 8 (19%)
- 3 (7%)
Intrinsic resistance
N=7
Aki1 expression
2+ 4 (57%)
1+ 3 (43%)
- 0 (0%)
Acquired resistance
N=7
Aki1 expression
2+ 6 (86%)
1+ 1 (14%)
- 0 (0%)

B


Score 0
Score 1+
Score 2+
Normal bronchial epithelium