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Src mediates ERK reactivation in gefitinib resistance in non-small cell lung cancer

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Abstract

To study epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance mechanisms, we established a novel gefitinib-resistant lung cancer cell line derived from an EGFR-mutant non-small cell lung cancer cell line (PC-9) pretreated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (designated PC9-GR). We found that gefitinib substantially suppressed the EGFR signaling pathway, whereas ERK was reactivated after several hours in PC9-GR but not in PC-9. The combination of gefitinib with ERK inhibition (by U0126) restored gefitinib susceptibility in PC9-GR, but PI3K-Akt inhibition with LY294002 did not. Although the levels of phosphorylated Src were up-regulated simultaneously with ERK reactivation, neither ERK suppression using U0126 nor an ERK-specific siRNA induced Src phosphorylation. Furthermore, dual inhibition of EGFR and Src restored gefitinib sensitivity in PC9-GR *in vitro* and *in vivo*. In conclusion, our results indicate that Src-mediated ERK reactivation may play a role in a novel gefitinib resistance mechanism, and that the combined use of gefitinib with a Src inhibitor may be a potent strategy to overcome this resistance.

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NSCLC, non-small cell lung cancer; PI, propidium iodide; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; TKI, tyrosine kinase inhibitor.

Key words: EGFR, Src, ERK, lung cancer, gefitinib, resistance

Introduction

Lung cancer remains a leading cause of cancer mortality worldwide. Non-small cell lung cancer (NSCLC) harboring somatic mutations in the gene encoding epidermal growth factor receptor (EGFR) is highly sensitive to EGFR-tyrosine kinase inhibitors (TKIs). Somatic EGFR mutations in NSCLC, including a 15-base pair in-frame deletion in exon 19 and L858R mutation in exon 21, contribute to EGFR-TKI sensitivity [1-3]. EGFR-TKIs compete with ATP for binding to the ATP-binding pocket of the tyrosine kinase receptor and demonstrate substantial inhibition of survival signals. Although EGFR-TKIs cause an immediate and dramatic clinical response, almost all patients with EGFR mutations who initially respond to EGFR-TKIs eventually develop acquired resistance, typically after around 1 year [4,5].

Recently, mechanisms of resistance to EGFR-TKIs have been identified. In approximately half of EGFR-TKI-resistant patients, resistance is associated with the emergence of a single missense mutation in exon 20 of EGFR that substitutes methionine for threonine at position 790 (“T790M”) in the kinase domain of the protein [6,7]. Irreversible EGFR-TKIs such as afatinib, HKI-272, and CL-387,785 have been considered to be potent for overcoming such resistance [8-10]. However, no clinical drug is currently available. MET amplification accounts for 20% of resistance

mechanisms [11]. Furthermore, Guix *et al*. [12] reported that insulin-like growth factor-1 receptor (IGF-1R) signaling mediated acquired resistance to EGFR-TKIs in A431 squamous cell lines with wild-type EGFR amplification. On the other hand, intrinsic resistance to EGFR-TKIs involves KRAS [13]. Although resistance to EGFR-TKIs in NSCLC has increased clinically, the resistance mechanisms are incompletely understood.

Many studies have demonstrated an association between smoking and poor prognosis in NSCLC patients, and smoking status was identified as an independent negative prognostic factor for survival in NSCLC patients treated with chemotherapy [14-17]. Furthermore, even in NSCLC patients treated with EGFR-TKIs, current and ex-smokers have a significantly poorer response rate or shorter survival time compared with light- or never-smokers [18-20]. However, the effects of smoking on EGFR-TKI resistance have not been examined. In this report, we describe a novel mechanism of gefitinib resistance induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone ("nicotine-derived nitrosamine ketone," NNK), a tobacco-specific nitrosamine.

Materials and Methods

Establishment of a gefitinib-resistant cell line

The “PC-9” cell line, which was derived from an untreated Japanese patient with a pulmonary adenocarcinoma carrying an in-frame deletion in EGFR exon 19 (del_E746-A750), shows strong sensitivity to EGFR-TKIs (e.g., gefitinib and erlotinib) [21]. PC-9 cells were initially treated with NNK (2.5 mg/mL) for 24 h and then washed with phosphate-buffered saline twice. The cells were then incubated in RPMI 1640 medium with gefitinib (0.5 μM) for 1 week. Before subsequent subcloning in RPMI 1640 medium with gefitinib (from 0.3 to 0.5 μM), surviving cells were cultured without gefitinib for 14 days. Finally, a gefitinib-resistant clone was identified, which was designated as “PC9-GR”. RPC-9, derived from PC-9 as described previously [22], harbors both a sensitive EGFR mutation and a secondary EGFR-TKI-resistant T790M mutation in EGFR exon 20.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Dose-response curves were determined using MTT assays. Briefly, cells were placed in 96-well plates at 1,500/well and exposed continuously to each drug for 96 h, and then quantified spectrophotometrically at 570 nm using a microplate reader (Bio-Rad, Hercules, CA). The drug concentration required to inhibit the growth of the tumor cells

by 50% (IC_{50}) was used to evaluate the effect of the drug. Each assay was performed at least in triplicate. All IC_{50} values are presented as means±standard deviation (SD).

Apoptotic morphology using Hoechst 33342 and propidium iodide (PI) differential staining

Cells were stained with Hoechst 33342 and PI (Invitrogen, Carlsbad, CA), and those with fragmented or condensed nuclei were defined as apoptotic cells. At least ten visual fields were observed under a fluorescence microscope for each sample and at least 500 cells were counted to determine the proportions of apoptotic and necrotic cells.

mRNA and genomic DNA extraction and analyses

Total mRNA was obtained using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, 1 μg of RNA was reverse-transcribed using Super Script III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Genomic DNA was extracted using a DNeasy Mini Kit (Qiagen). cDNA or genomic DNA was amplified and analyzed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) by real-time quantitative polymerase chain reaction using a GeneAmp 5700 apparatus (Applied Biosystems). Target gene-specific primers were designed using the

web tool “Primer3” (http://primer3.sourceforge.net). The sequences are available on request. The concentration of DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) in Okayama University Medical School’s Central Research Laboratory (Okayama, Japan).

Immunoblotting analysis

Cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerolphosphate, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor tablets [Roche Applied Sciences, Indianapolis, IN]) and the proteins were then separated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes, and probed with specific antibodies. Peroxidase-labeled anti-rabbit or anti-mouse antibodies (GE Healthcare Biosciences, Piscataway, NJ) were used as the secondary antibody followed by detection with an Enhanced Chemiluminescence Plus kit (GE Healthcare Biosciences).

Reagents and antibodies

Gefitinib was purchased from Tocris Bioscience (Ellisville, MO). NNK was purchased

from TRONTO Research (North York, ON, Canada). LY294002 (PI3K inhibitor; #9901) and U0126 (MEK inhibitor; #9903) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit antisera against EGFR, phospho-EGFR (pY1068), p44/42 mitogen-activated protein kinase (MAPK) (ERK1/2), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), Akt, phospho-Akt (pSer473), phospho-MET (pMET; pY1234/1235), IGF-1R, phospho-IGF-1R (pIGF-1R; pY1131), "phosphatase and tensin homolog deleted on chromosome 10" (PTEN), Src, phospho-Src, Stat-3, phospho-Stat-3, cyclooxygenase-2 (COX-2), and β-actin were purchased from Cell Signaling Technology. Polyclonal antibodies against MET were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-labeled anti-rabbit or anti-mouse antibodies (GE Healthcare Biosciences) were used as secondary antibodies.

Small interfering RNA (siRNA) experiments

Approximately 2×10^5 cells were plated in six-well plates in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. After 24 h, the cells were transfected with an siRNA oligonucleotide or negative control siRNA using Lipofectamine RNAiMAX (Invitrogen) at a final RNA concentration of 30 nM in serum-free Opti-MEM (Invitrogen) medium according to the manufacturer's protocol. At 24 h after transfection,

we changed the medium to normal conditioned medium. After an additional 24 h, the cells were lysed and extracted as above. Immunoblotting was performed as described above. The siRNA oligonucleotides for Src and ERK (predesigned siRNA, ID number SASI_Hs01_00112907 Validated MISSION siRNA and SASI_Hs01_00190617 Validated MISSION siRNA, respectively), and the negative control siRNA (Mission_Negative Control SIC-001), which does not induce non-specific effects on gene expression, were purchased from Sigma-Aldrich (St. Louis, MO).

Enzyme immunoassay

Cells were plated at a density of 3.0×10^5 cells/well in 6-well plates 24 h prior to experiments. Cells were exposed to 0.01% DMSO or 1 μM of gefitinib for 12 h. Then, culture medium in each well was collected and centrifuged. Supernatants were assayed for released Prostaglandin E2 (PGE2) using PGE2 Express EIA Kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions.

Xenograft model

Female athymic mice, 7 weeks of age, were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). All mice were provided with sterilized food and water

and housed in a barrier facility under a 12-h/12-h light/dark cycle. Cells (2×10^6) were injected subcutaneously into the backs of the mice. At 1 week after injection, the mice were randomly assigned to one of four groups (6 or 7 mice per group); the groups were administered vehicle, 5 mg/kg/day of gefitinib, 15 mg/kg/day of dasatinib, and 5 mg/kg/day of gefitinib plus 15 mg/kg/day of dasatinib, respectively. Vehicle, gefitinib, and dasatinib were administered by gavage once per day, five times per week. Tumor volume (width × width × length/2) was determined periodically. All tumor volumes are expressed as means±SD. Differences in tumor volume were evaluated using Student's *t*-test. The tumor volume percent was determined from the change in average tumor volume for each treated group relative to the vehicle-treated group in the case of tumor regression.

All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University (Okayama, Japan).

Statistical analyses

For the experimental data, all P-values correspond to two-sided tests, with the significance set at $P < 0.05$. Statistical analyses were conducted using STATA software

(ver. 10; StataCorp, College Station, TX).

Results

Continuous exposure to gefitinib with NNK induced resistance to gefitinib

To establish gefitinib-resistant cell lines, we used PC-9 cells pretreated with NNK followed by gefitinib as described in the Materials and Methods. The doubling times for PC-9 (23.0 h) and PC9-GR (21.7 h) were similar. The IC_{50} value of PC9-GR for gefitinib was approximately 2.60±0.88 μM, which was a 124-fold decrease in gefitinib sensitivity compared with the parental line PC-9 (IC_{50}: 0.021±0.0084 μM; Fig. 1A). The growth inhibitory effect of gefitinib on PC-9 and PC9-GR was confirmed by cell counting assays (Fig. S1A). Staining with Hoechst 33342 and PI showed that treatment with gefitinib induced apoptosis in PC-9 to a greater extent than in PC9-GR (Fig. S1B and S1C). PC9-GR showed cross-resistance to erlotinib (IC_{50} values: 0.0073±0.0014 μM in PC-9 vs. 1.50±0.50 μM in PC9-GR).

Peptide nucleic acid-locked nucleic acid PCR clamp methods and RT-PCR confirmed that PC9-GR had neither the secondary T790M mutation in EGFR exon 20 nor MET gene amplification (Fig. S2A and S2B). Western blotting demonstrated that

IGF-1R signaling was not activated in PC9-GR (Fig. S2C). The PTEN expression levels were also similar in both cell lines (Fig. S2D). A direct sequence analysis of genomic DNA revealed that a wild-type KRAS gene was present in PC9-GR. We also found no apparent mutation in other EGFR signaling pathways (KRAS, BRAF, c-Raf, and MEK1/2) by direct sequence analysis (data not shown).

ERK phosphorylation in PC9-GR cells was maintained in the presence of gefitinib

Protein expression in PC-9 and PC9-GR is shown in Fig. 1B. The levels of phosphorylated EGFR, AKT, and ERK in PC9-GR were increased compared with those in PC-9 treated without gefitinib. Treatment for 24 h with gefitinib ($\geq$ 0.1 μM) of both the parental and resistant cells inhibited the phosphorylation of EGFR and of the downstream factors Akt and ERK, although pERK was still maintained in PC9-GR. That is, gefitinib inhibited ERK activation in accordance with the suppression of pEGFR in PC-9, whereas ERK was still activated in PC9-GR.

PC9-GR cell growth was significantly suppressed by a MEK inhibitor but not a PI3K inhibitor

Protein expression was assessed with a MEK inhibitor (U0126) or a PI3K inhibitor

(LY294002) in combination with gefitinib. ERK phosphorylation was completely suppressed by the combination of gefitinib with U0126, even in PC9-GR (Fig. 2A). Likewise, the combination of gefitinib with LY294002 suppressed EGFR and Akt activation in the resistant cells (Fig. 2B). The growth of PC9-GR was significantly suppressed when treated with the combination of gefitinib and U0126 (Fig. 2C), whereas the combination of gefitinib and LY294002 showed marginal growth inhibition in PC9-GR (not statistically significant; Fig. 2D). The results suggested that bypassing signals in PC9-GR may depend mainly on the EGFR-ERK pathway.

Time-dependent ERK reactivation in PC9-GR is correlated with Src activation

Cells were exposed to 2 μM gefitinib for the indicated time (Fig. 3A). EGFR and Akt phosphorylation was down-regulated by gefitinib in a time-dependent manner. Although gefitinib immediately suppressed ERK phosphorylation in both cell lines, ERK was reactivated after 6 h of exposure to gefitinib in PC9-GR. Interestingly, Src activation was observed in accordance with ERK reactivation in PC9-GR. Densitometry confirmed that Src phosphorylation, normalized to GAPDH, increased significantly in the resistant cells (Fig. S3A). Conversely, downstream ERK inhibition by U0126 and by siRNA did not induce Src activation in either cell line (Fig. 3B and 3C). Taken together,

ERK reactivation was apparently induced by Src activation.

The combination of gefitinib with Src inhibition, using dasatinib or siRNA, was effective against PC9-GR

Suppression of Src phosphorylation by treatment with gefitinib and dasatinib, a Src inhibitor, resulted in ERK suppression and significant inhibition of cell proliferation (Fig. 4A and 4B). Dose-response curves for gefitinib with or without dasatinib are shown in Fig. 4C. The IC_{50} values for gefitinib were decreased significantly in PC9-GR (3.5 μM without dasatinib vs. 0.008 μM with dasatinib). Furthermore, an siRNA specific for Src showed significantly increased gefitinib sensitivity in PC9-GR (IC_{50} values: 5.3 μM without siRNA vs. 0.47 μM with siRNA; Fig. 4D and 4E). The Src knockdown did not affect pERK expression (Fig. 4D), consistent with the result of Src inhibition with dasatinib (Fig. 4A). These results suggest that dual inhibition of EGFR and Src may overcome gefitinib resistance.

Effect of combined gefitinib and dasatinib in a mouse xenograft model with PC9-GR

To extend our findings to *in vivo* models, we conducted xenograft studies in athymic

nude mice injected with PC-9 and PC9-GR. First, we examined xenograft tumors to determine the degree of gefitinib resistance in PC9-GR. In the 5 mg/kg gefitinib-treated group, PC9-GR xenograft tumors were able to grow compared to the vehicle group, whereas parental PC-9 xenograft tumors were not (Fig. S4). Subsequently, we investigated whether the combination of gefitinib with dasatinib had a synergistic effect on the growth of PC9-GR. At 7-10 days after tumor cell injection, the mice were divided into four groups and treated with vehicle, gefitinib, dasatinib, or a combination of both drugs by oral gavage for 45 days. The combined treatment showed growth inhibition of the PC9-GR xenograft tumor (Fig. 5), consistent with our *in vitro* results. The tumor volume percentages were 103.5% in the gefitinib arm, 84.7% in the dasatinib arm, and 50.9% in the combined arm on day 45. However, there was no statistically significant difference in tumor size between combination therapy and gefitinib monotherapy ($P = 0.067$).

COX-2 and PGE2 levels in PC-9 and PC9-GR

COX-2 expression in PC-9 and PC9-GR cells did not differ irrespective of gefitinib-treatment (Fig. S5A). Subsequently, PGE2 production was assessed by enzyme immunoassay (Fig. S5B). PGE2 levels were also similar in PC-9 and PC9-GR cells

irrespective of gefitinib-treatment.

Discussion

We established a novel EGFR-TKI-resistant cell line in which the resistance mechanism involved ERK reactivation mediated by Src. A role for Src in resistance to EGFR-TKIs has not been reported previously. In this study, NNK exposure followed by gefitinib treatment induced resistance to gefitinib in the EGFR-mutated cells. PC9-GR was sensitive to gefitinib when combined with a Src inhibitor.

Koizumi *et al.* [23] reported gefitinib-resistant cells using MNNG, a known DNA alkylator. Resistance in their cells was associated with MET amplification. We established the PC9-GR line using NNK according to a similar procedure, but the cells did not harbor acquired resistance mechanisms, including T790M and MET amplification, or intrinsic resistance mechanisms, including KRAS mutations and a loss of PTEN. It is unclear whether the difference in DNA alkylating agents induced different resistance mechanisms. Gefitinib-resistant cell lines have been established traditionally by step-wise, chronic exposure to gefitinib, from low to high concentrations, for up to 6 months [11,12,22] whereas PC9-GR acquired strong resistance to gefitinib within 2 months. One explanation for the rapid emergence of

gefitinib resistance in NNK-pretreated PC9-GR cells is the activation of an alternative survival signaling pathway. To date, such alternative pathways have been reported. Tobacco smoke was reported to cause an increase in COX-2 and its downstream product, prostaglandin E2, in normal and transformed oral epithelial cells [24]. Among the various downstream products of COX-2, thromboxane A(2) and thromboxane B(2), induced by NNK administration, were observed in lung cancer cell lines as well as in lung cancer tissues of smokers. They concluded that the increased thromboxane A(2) activated “cyclic adenosine monophosphate response element binding protein” (CREB) through the PI3K/Akt and ERK pathways [25]. In our study, there was no significant difference in COX-2 expression or PGE2 production between PC-9 and PC9-GR cells (Fig. S5). NNK-induced pulmonary adenocarcinoma in hamsters also showed overexpression of β2-adrenergic receptor pathway components, including CREB, and EGFR pathway components, including Raf-1 and ERK1/2 [26]. Together, these results suggest that NNK induced various alternative survival signals and led to the acquisition of gefitinib resistance, which was mediated by bypass signals, as seen in our study. Since we could not explain the reason why 24 h-exposure of NNK contributed to increased activation of Src-ERK axis in PC9-GR cells, further study was needed for the elucidation of the mechanism.

The relationship between smoking status and prognosis in lung cancer patients has long been evaluated. Doll and Hills [27] first reported a dose-response relationship between the number of cigarettes smoked and lung cancer death rates 50 years ago. Since then, smoking has been associated with cancer risk, including not only lung cancer, but also oral, laryngeal, esophageal, gastric, and colorectal cancers [28-30]. Many reports have shown that heavy smoking status is associated with a poor prognosis in patients with NSCLC [14-17]. Additionally, the longer the time since the cessation of smoking at treatment initiation, the better the survival outcome and recurrence-free survival in early-stage NSCLC patients [31]. However, a recent prospective study in chemo-naïve patients with advanced NSCLC demonstrated that smoking during treatment with cytotoxic agents was not significantly associated with survival [32]. The anti-tumor effects of EGFR-TKIs such as gefitinib and erlotinib are affected by smoking status. Never smoking or light smoking is thought to be more beneficial for patients treated with gefitinib and an independent negative predictor of gefitinib treatment [19,33-35]. In a large-scale phase III study and a global phase IV post-marketing surveillance study, current or past smoking was determined to be a negative predictive marker for treatment response and longer survival [18,20]. Thus, the negative effect of smoking on EGFR-TKI treatment has been discussed. However, few reports have

described the influence of smoking on drug resistance because of the complexity of the ingredients in cigarette smoke (CS); there are many chemicals and carcinogens in addition to nicotine that are associated with addiction. Indeed, over 4,000 chemical compounds are created by burning a cigarette, and 69 of those are known to cause cancer [36]. NNK has been widely recognized to be strongly associated with the induction of tobacco-related carcinogenesis [38,39] or somatic mutations in KRAS associated with *de novo* resistance to EGFR-TKIs [13]. KRAS mutations in codons 12 and 13 (G-to-T transversions and G-to-A transitions) occur in 15-25% of NSCLC patients, especially in adenocarcinoma, and are strongly associated with cigarette smoking history in NSCLC [39,40]. Some have argued, based on meta-analyses, that KRAS mutations represent a strong candidate predictive biomarker for non-responsiveness to both monoclonal antibodies and EGFR-TKI-based strategies [41,42]. Although PC9-GR did not carry a KRAS mutation, other bypassing signals related to NNK may be considered.

The signal transduction pathway activating ERK via Src remains to be determined. A recent study showed that the "receptor activator of nuclear factor-κB ligand" (RANKL)/RANK pathway plays an important role in tumor migration, metastasis, proliferation, and tumorigenesis *in vitro* and *in vivo*, in mammary tumors

[43,44]. They demonstrated that stimulation by RANKL resulted in activation of MAPKs such as ERK, p38MAPK, and JNK, and the combination of specific inhibitors of MAPKs suppressed RANKL-induced cell migration. Moreover, MAPK phosphorylation could be blocked by a specific Src inhibitor and by a Src-specific siRNA [44]. The Src family of protein tyrosine kinases (SFK) cooperates with multiple receptor tyrosine kinases such as EGFR and modulates various signaling pathways, including those involved in survival, growth, and transformation, in cancer cells [45]. Elevated SFK activity has been reported in various human tumors, including lung cancer [46,47], and Src inhibitor monotherapy caused growth inhibition and the induction of cell death in EGFR-driven NSCLC [48,49]. To date, synergistic interactions between EGFR and Src in mitogenesis and tumorigenesis have been established *in vitro* [50]. In breast cancer, co-overexpression of both EGFR and Src occurs in a subset of cell lines and tumor tissues [51]. These results suggest a connection between EGFR and Src, suggesting the combination of EGFR-TKIs with Src inhibitors as a potential therapeutic strategy. To date, an activating mutation in Src has not been reported in NSCLC, but Src-amplified NSCLC cell lines show strong sensitivity to Src inhibitors [52]. As reported previously, the amplification of target genes can predict susceptibility to target-specific treatment in ERBB2-amplified breast

cancer and EGFR-amplified lung cancer [53,54]. Cooperation between EGFR and Src has been demonstrated in many tumors, notably in head and neck cancer and NSCLC [55,56]. EGFR-mutated NSCLC demonstrates differential sensitivity to Src inhibitors. Specifically, S768I and L861Q mutants are less sensitive to Src inhibition, whereas L858R or exon 19 deletion mutations are more sensitive to Src inhibitors. Interestingly, an exon 19 deletion/T790M double mutant remained sensitive to Src inhibitors whereas an L858R/T790M double mutant did not [57]. These differences in sensitivity to Src inhibitors have not been fully characterized. A phase I/II study of the combination of erlotinib with dasatinib in unselected NSCLC patients has already been conducted [58]. In that study, the overall disease control rate was 62% with a median progression-free survival of 2.7 months and overall survival of 5.6 months. If an EGFR-TKI refractory tumor demonstrated a resistance mechanism such as that in PC9-GR, the combination of an EGFR-TKI with a Src inhibitor may be a potent treatment strategy.

Recently, Ercan *et al*. [59] reported a novel EGFR-TKI resistance mechanism. They generated WZ4002 (irreversible EGFR-TKI)-resistant cell lines (PC9 WZR), which harbored an active mutation (del_E746-A750 in EGFR exon 19) and an EGFR-TKI-resistant mutation (T790M in exon 20). The resistant cells demonstrated sustained ERK phosphorylation in the presence of WZR4002, as seen in our resistant

cells. Although the resistant cells contained *MAPK1* amplification compared with the parental cells, and the resistance could be overcome by the inhibition of ERK1/2, they further demonstrated that down-regulation of DUSP6, a negative regulator of MAPK signaling, induced subsequent activation of ERK1/2 signaling. Although we investigated DUSP6 expression in our cell lines, DUSP6 protein expression was comparable between the parental and resistant cells (Fig. S3B). More recently, Filosto *et al*. [60] described transient EGFR-TKI resistance caused by Src activation in wild-type or L858R mutant EGFR NSCLC cell lines exposed to CS for a short time. They demonstrated that CS exposure induced an aberrant interaction between Src and EGFR, and, when treated with PP1 and PP2 (Src-specific inhibitors), Src-dependent trans-phosphorylation at Y845 of EGFR was markedly inhibited. Furthermore, transient EGFR-TKI resistance was overcome when combined with Src-specific inhibitors. They concluded that CS-induced oxidative stress evoked this EGFR-TKI resistance. Although their model emphasized Src function in EGFR-TKI resistance, similar to our study, we specifically used NNK, an abundant cigarette ingredient, and established a resistant cell line.

Our study has some limitations. First, we investigated only one cell line. Although Src-mediated ERK reactivation may indeed be a resistance mechanism, we

must investigate other EGFR-mutated cell lines, which could have as-yet unknown resistance mechanisms. Second, the NNK-induced resistance model in the present study is difficult to apply immediately to the clinical situation in patients with EGFR mutations. Although no clinical sample harboring this resistance mechanism has been reported, it must be noted that Src could play a role in EGFR-TKI resistance in some situations. Third, we unfortunately failed to show the superiority of combination treatment with gefitinib and dasatinib over each monotherapy with statistical significance in xenograft model ($P = 0.067$). The reason might be because there were comparatively small numbers (6 or 7 mice per group) or issues of the chemicals such as treatment doses or schedules. The kinetics of the two drugs administered concomitantly remains unclear *in vivo*, which applied to the schedule of the treatment as well. Otherwise the combination efficacy might not be acknowledged *in vivo* in fact. Further investigation is necessary to clarify it.

In conclusion, our results indicate that Src-mediated ERK reactivation may play a role in a novel gefitinib resistance mechanism. The combination of gefitinib with a Src inhibitor may be a potent strategy for overcoming this resistance.

Figure legends

Fig. 1 - Effects of gefitinib on PC-9 (parent) and PC9-GR (resistant). (A) Dose-response curves for the two cell lines. Growth inhibition was determined by MTT assays. (B) Protein expression was determined by Western blotting.

Fig. 2 - Protein expression and cell growth in the presence of gefitinib and MEK or PI3K inhibitors. Protein expression in PC-9 and PC9-GR in the presence of gefitinib and U0126 (A) or LY294002 (B). The growth of PC-9 and PC9-GR was assessed following treatment with gefitinib and U0126 (C) or LY294002 (D). Cells were seeded at 50,000/well and incubated in the presence of the indicated drug for 72 h at 37°C in a 5% CO_2 incubator. After 72 h of treatment, the cells were counted. Bars indicate SDs. * $P < 0.05$. The concentrations of gefitinib, U0126, and LY294002 were 0.5, 10, and 10 μM, respectively.

Fig. 3 - Time-dependent ERK reactivation in PC-9 and PC9-GR. (A) Protein expression in the presence of gefitinib (2 μM) at the indicated times. ERK inhibition by U0126 (B) and an ERK-specific siRNA (C) did not induce Src phosphorylation.

Fig.4 - Protein expression (A) and cell survival (B) of PC-9 and PC9-GR treated with gefitinib and dasatinib for 24 h. (C) Dose-response curves were determined using MTT assays. Cells were treated with gefitinib alone or combined with dasatinib. (D) Protein expression of Src following treatment with Src-specific siRNA. The Src knockdown did not affect pERK expression, consistent with the result of Src inhibition with dasatinib. (E) Dose-response curves of gefitinib in PC-9 and PC9-GR pre-treated with Src-specific siRNA. Abbreviations: gefitinib +, 0.5 μM gefitinib; dasatinib +, 50 nM dasatinib; dasatinib ++, 100 nM dasatinib.

Fig. 5 - Combined effects of gefitinib with dasatinib in an *in vivo* model. Combination treatment was more effective than either agent (gefitinib or dasatinib) alone in the PC9-GR xenograft model. The numbers to the right of the curves are the percent tumor volumes determined from the change in average tumor volume in each treated group relative to the vehicle-treated group. Abbreviations: v, vehicle; G5, 5 mg of gefitinib; D15, 15 mg of dasatinib.

Supplemental figure legends

Fig. S1 - (A) The growth inhibitory effects of gefitinib on PC-9 and PC9-GR were confirmed by cell counting assays. * $P < 0.05$. (B) Hoechst 33342 and PI differential staining is shown in fluorescent micrographs. (a) PC-9 cells were treated without gefitinib for 48 h as a control. (b) PC-9 cells treated with gefitinib for 48 h. (c) PC9-GR cells treated without gefitinib for 48 h. (d) PC9-GR cells treated with gefitinib for 48 h. White arrows and red arrowheads indicate apoptotic cells and necrotic cells, respectively. Magnification, ×600. (C) The results of (B) were visualized. The proportion of apoptotic cells was less frequent in PC9-GR.

Fig. S2 - Direct sequence analysis and real-time quantitative reverse transcription confirmed PC9-GR had neither (A) the secondary T790M mutation in EGFR exon 20, nor (B) MET amplification. (C) Western blotting demonstrated that IGF-1R signaling was not activated in PC9-GR. (D) Western blotting demonstrated PTEN expression in both cell lines.

Fig. S3 - (A) Densitometry confirmed that Src phosphorylation, normalized to GAPDH,

was increased significantly in the resistant cells. (B) DUSP6 protein expression was comparable between PC-9 and PC9-GR.

Fig. S4 - Effects of gefitinib in a PC-9 xenograft model. Oral gefitinib (5 mg) showed substantial growth inhibition of the PC-9 xenograft. The numbers to the right of the curves are the percent tumor volumes determined from the change in the average tumor volume in each treated group relative to the vehicle-treated group.

Abbreviations: v, vehicle; G5, 5 mg of gefitinib; G10, 10 mg of gefitinib.

Fig. S5 - (A) COX-2 expression in PC-9 and PC9-GR cells was evaluated using Western blotting. (B) PGE2 concentration was assessed by enzyme immunoassay. Bar means ± SE. Each assay was done in triplicated.

References

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Fig.1

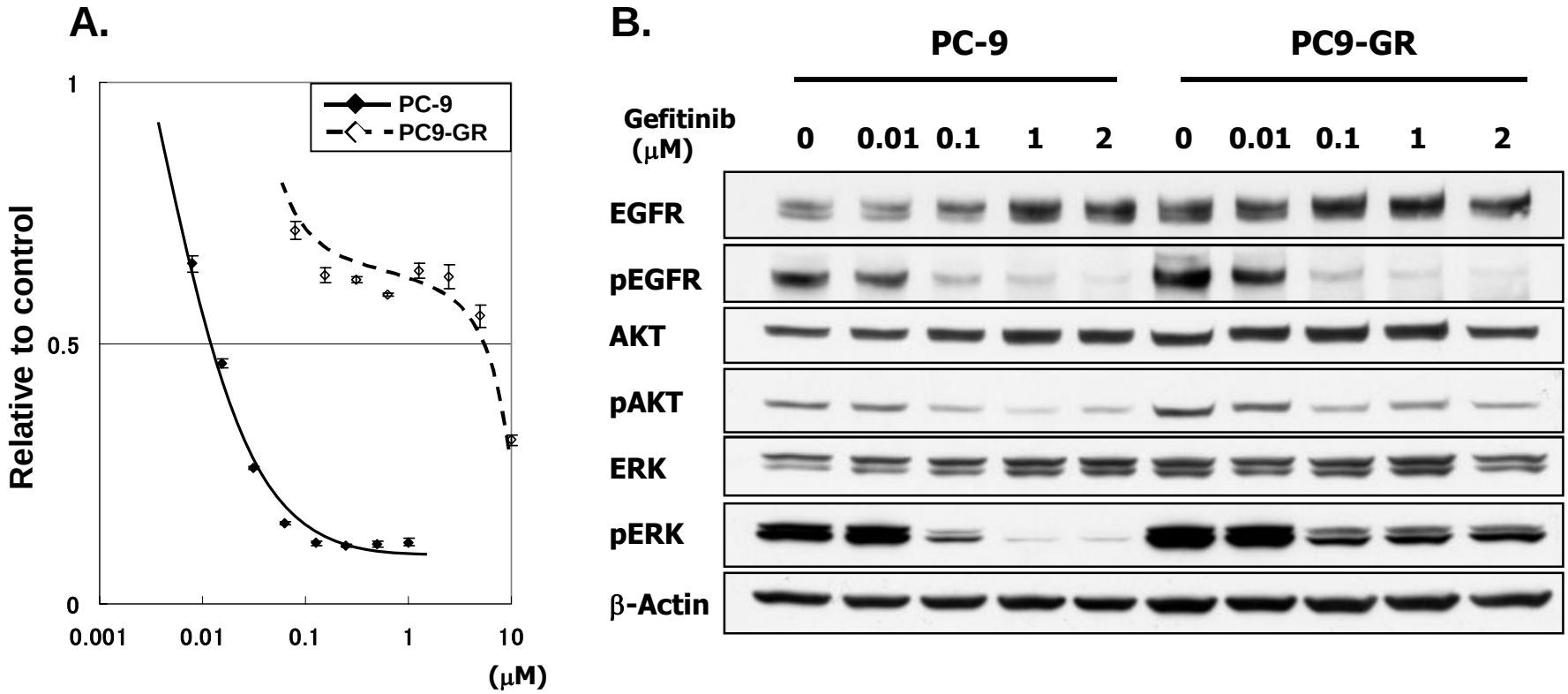

A.
PC-9
PC9-GR
Relative to control
1
0.5
0
0.001
0.01
0.1
1
10
(μM)
B.
PC-9
PC9-GR
Gefitinib (μM)
0
0.01
0.1
1
2
0
0.01
0.1
1
2
EGFR
pEGFR
AKT
pAKT
ERK
pERK
β-Actin

Fig.2

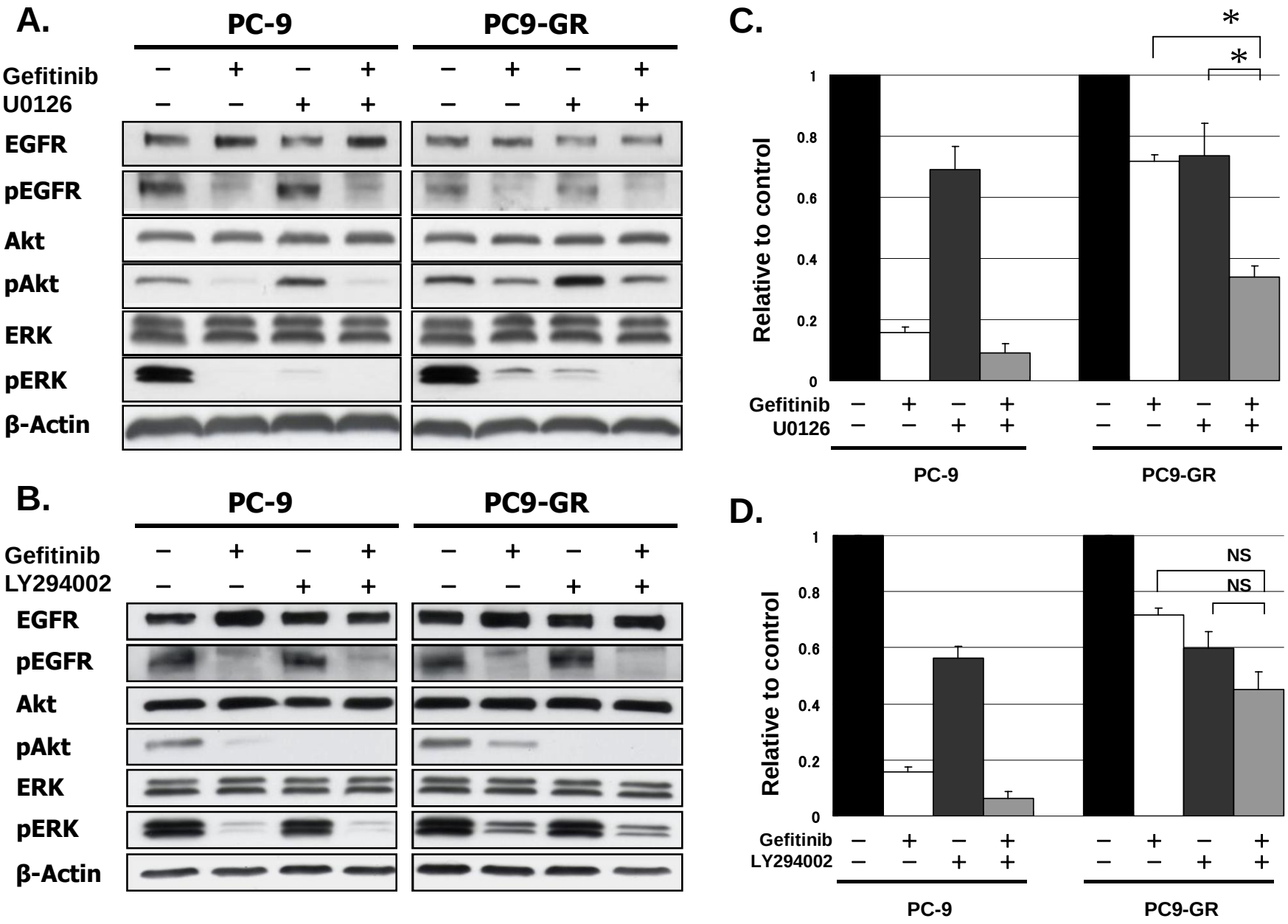

A.
PC-9
PC9-GR
Gefitinib
U0126
EGFR
pEGFR
Akt
pAkt
ERK
pERK
β-Actin
B.
PC-9
PC9-GR
Gefitinib
LY294002
EGFR
pEGFR
Akt
pAkt
ERK
pERK
β-Actin
C.
Relative to control
Gefitinib
U0126
PC-9
PC9-GR
*
*
D.
Relative to control
Gefitinib
LY294002
PC-9
PC9-GR
NS
NS

Fig.3

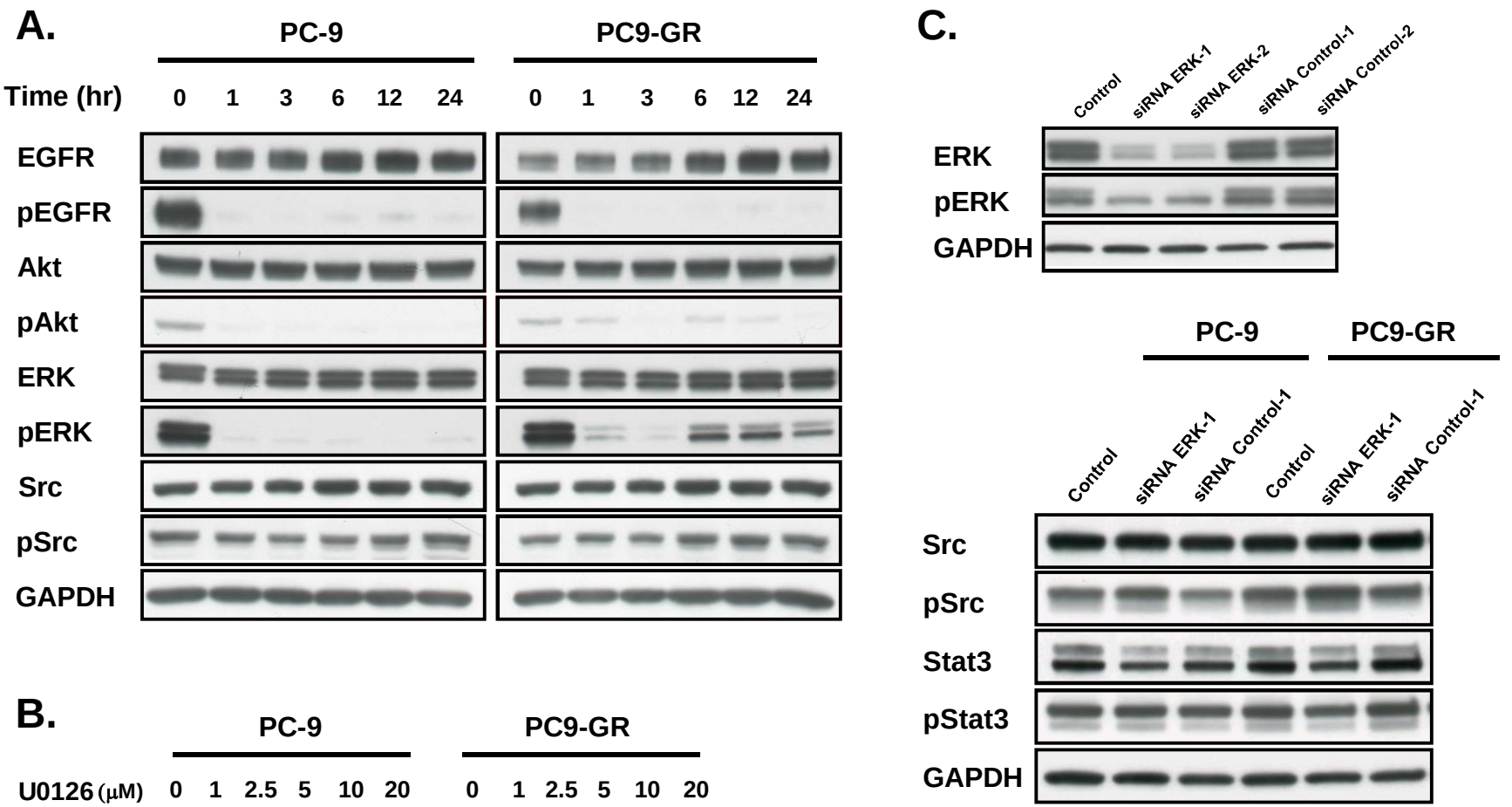

A.
PC-9
PC9-GR
Time (hr)
0 1 3 6 12 24
0 1 3 6 12 24
EGFR
pEGFR
Akt
pAkt
ERK
pERK
Src
pSrc
GAPDH
B.
PC-9
PC9-GR
U0126 (μM)
0 1 2.5 5 10 20
0 1 2.5 5 10 20
Src
pSrc
ERK
pERK
GAPDH
C.
Control
siRNA ERK-1
siRNA ERK-2
siRNA Control-1
siRNA Control-2
ERK
pERK
GAPDH
PC-9
PC9-GR
Control
siRNA ERK-1
siRNA Control-1
Control
siRNA ERK-1
siRNA Control-1
Src
pSrc
Stat3
pStat3
GAPDH

Fig.4

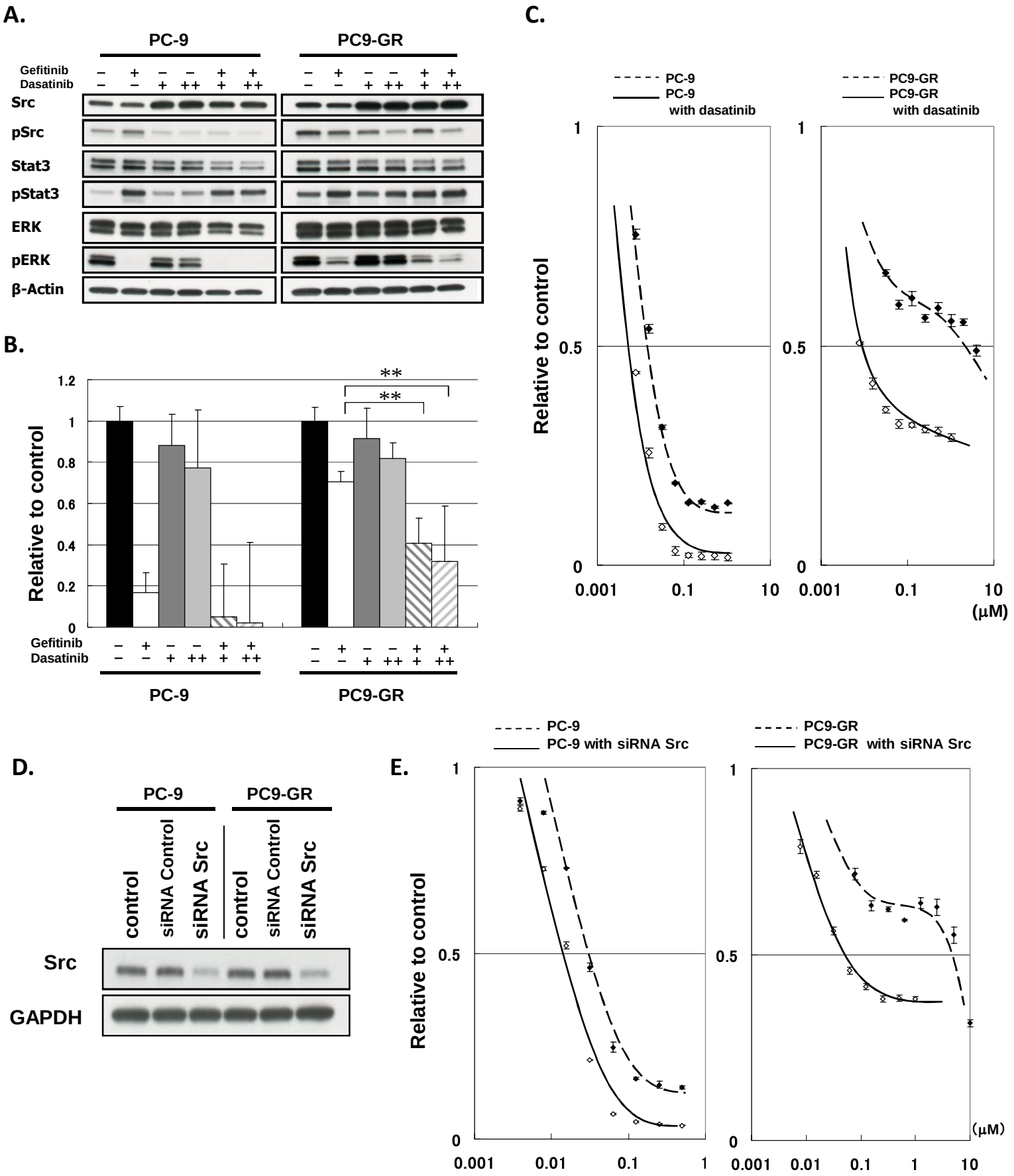

A.
PC-9
PC9-GR
Gefitinib
Dasatinib
Src
pSrc
Stat3
pStat3
ERK
pERK
β-Actin
B.
Relative to control
**
**
Gefitinib
Dasatinib
PC-9
PC9-GR
C.
PC-9
PC-9 with dasatinib
PC9-GR
PC9-GR with dasatinib
Relative to control
(μM)
D.
PC-9
PC9-GR
control
siRNA Control
siRNA Src
Src
GAPDH
E.
PC-9
PC-9 with siRNA Src
PC9-GR
PC9-GR with siRNA Src
Relative to control
(μM)

Fig.5

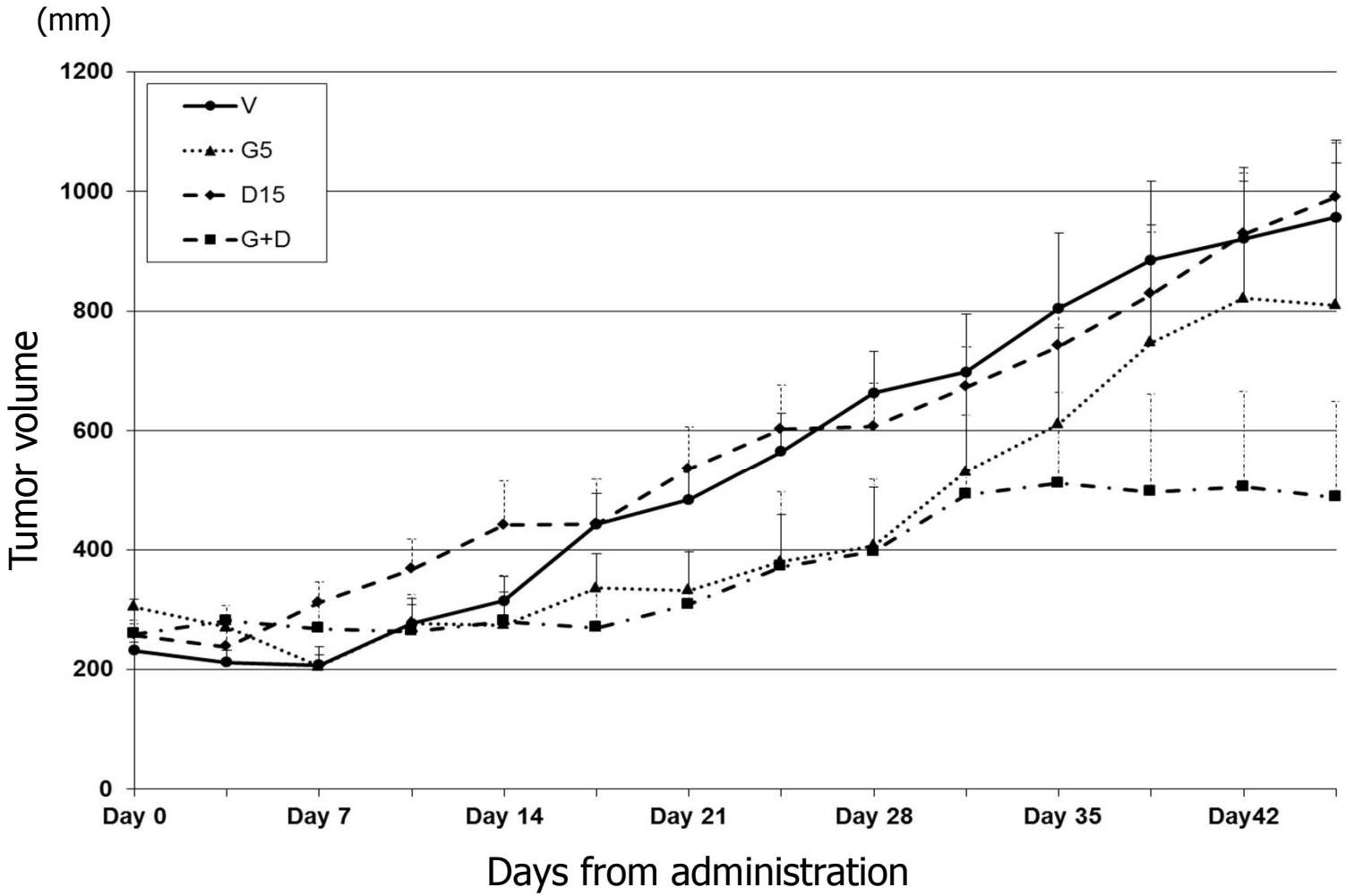
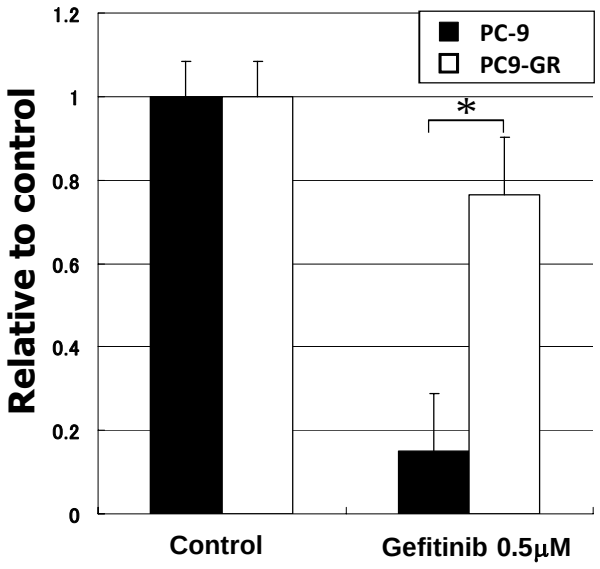
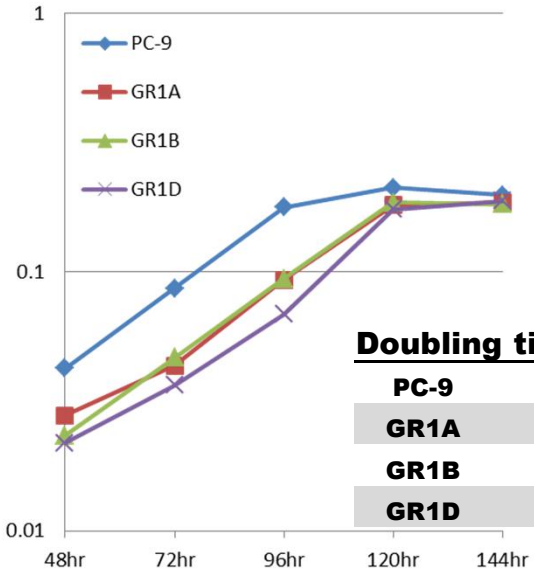

(mm)
1200
1000
800
600
400
200
0
V
G5
D15
G+D
Tumor volume
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day42
Days from administration

Figure S1

A.

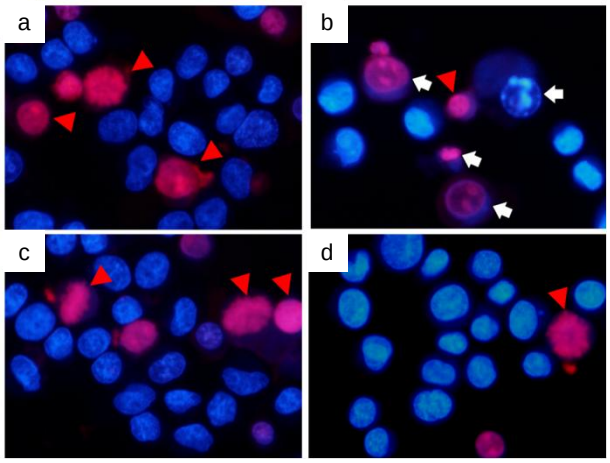


B.



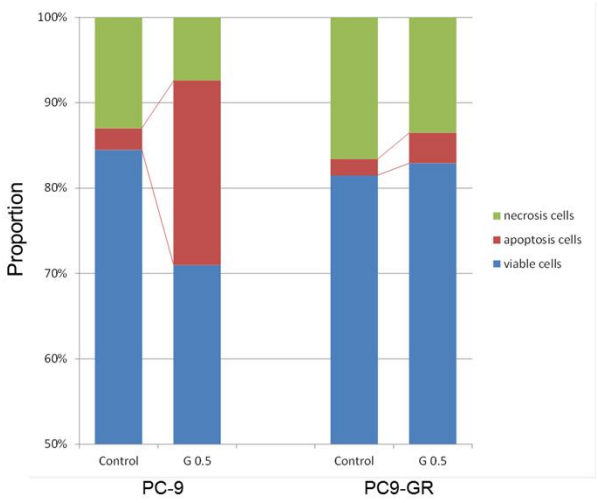
Doubling time (hr)	
PC-9	23.01
GR1A	21.73
GR1B	23.63
GR1D	26.42

C.



Apoptotic morphology
by Hoechst33342/PI double stain

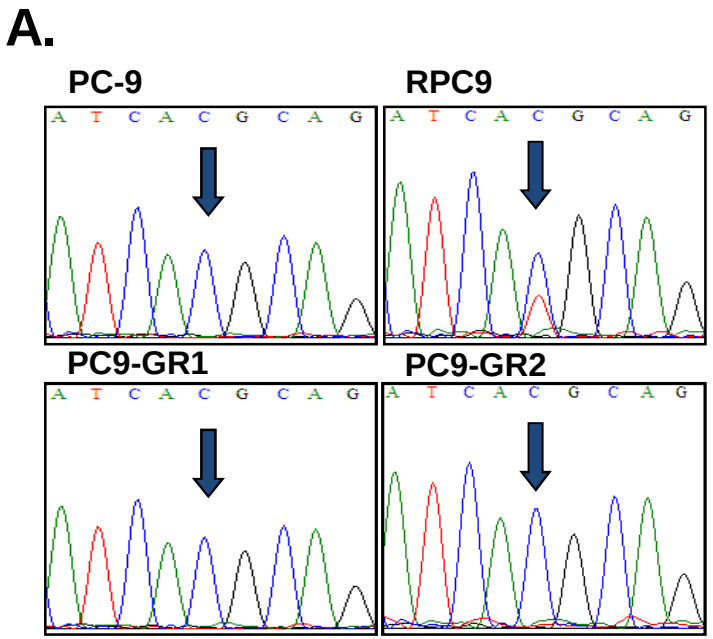
D.

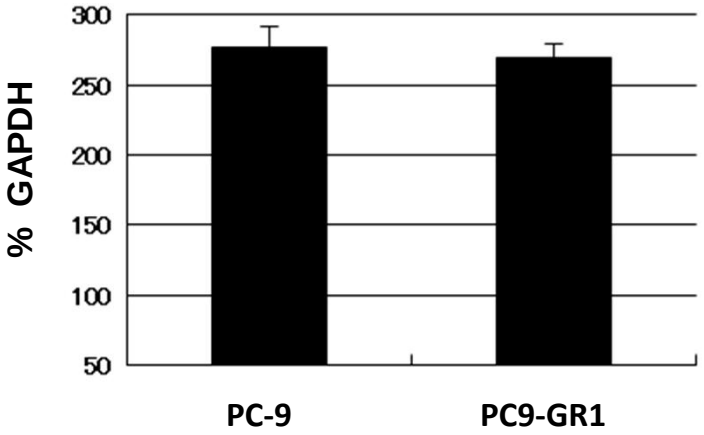


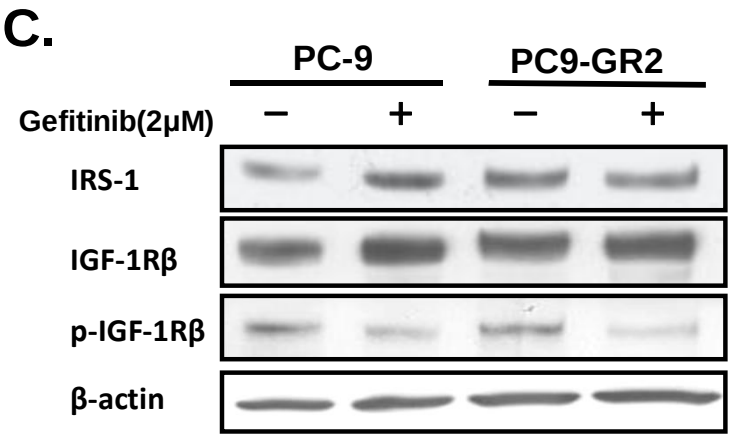
E.

	Gefitinib (μM)	Erlotinib (μM)	Vandetanib (μM)
PC-9	0.021±0.0084	0.0073±0.0014	0.12±0.018
PC9-GR	2.60±0.88	1.50±0.50	1.9±0.44

Fig. S2


A.
PC-9
RPC9
PC9-GR1
PC9-GR2
A T C A C G C A G


B.
% GAPDH
300
250
200
150
100
50
PC-9
PC9-GR1


C.
PC-9
PC9-GR2
Gefitinib(2μM)
–
+
–
+
IRS-1
IGF-1Rβ
p-IGF-1Rβ
β-actin

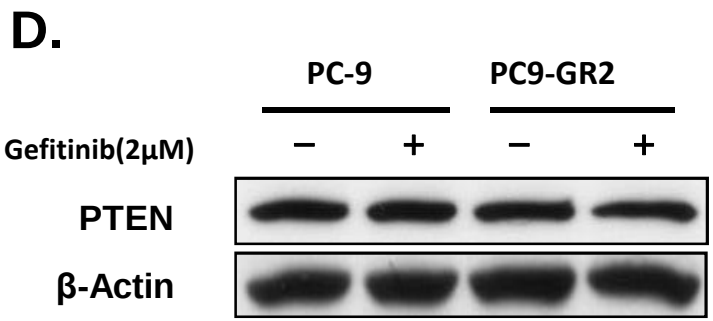

D.
PC-9
PC9-GR2
Gefitinib(2μM)
–
+
–
+
PTEN
β-Actin

Fig. S3

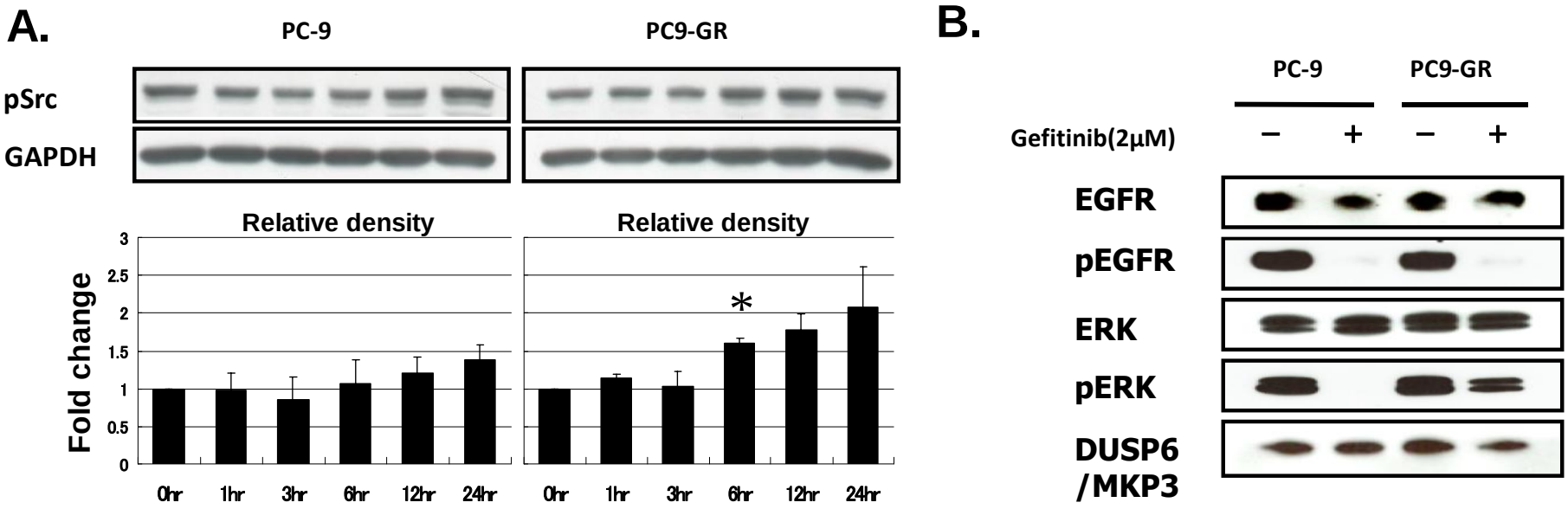
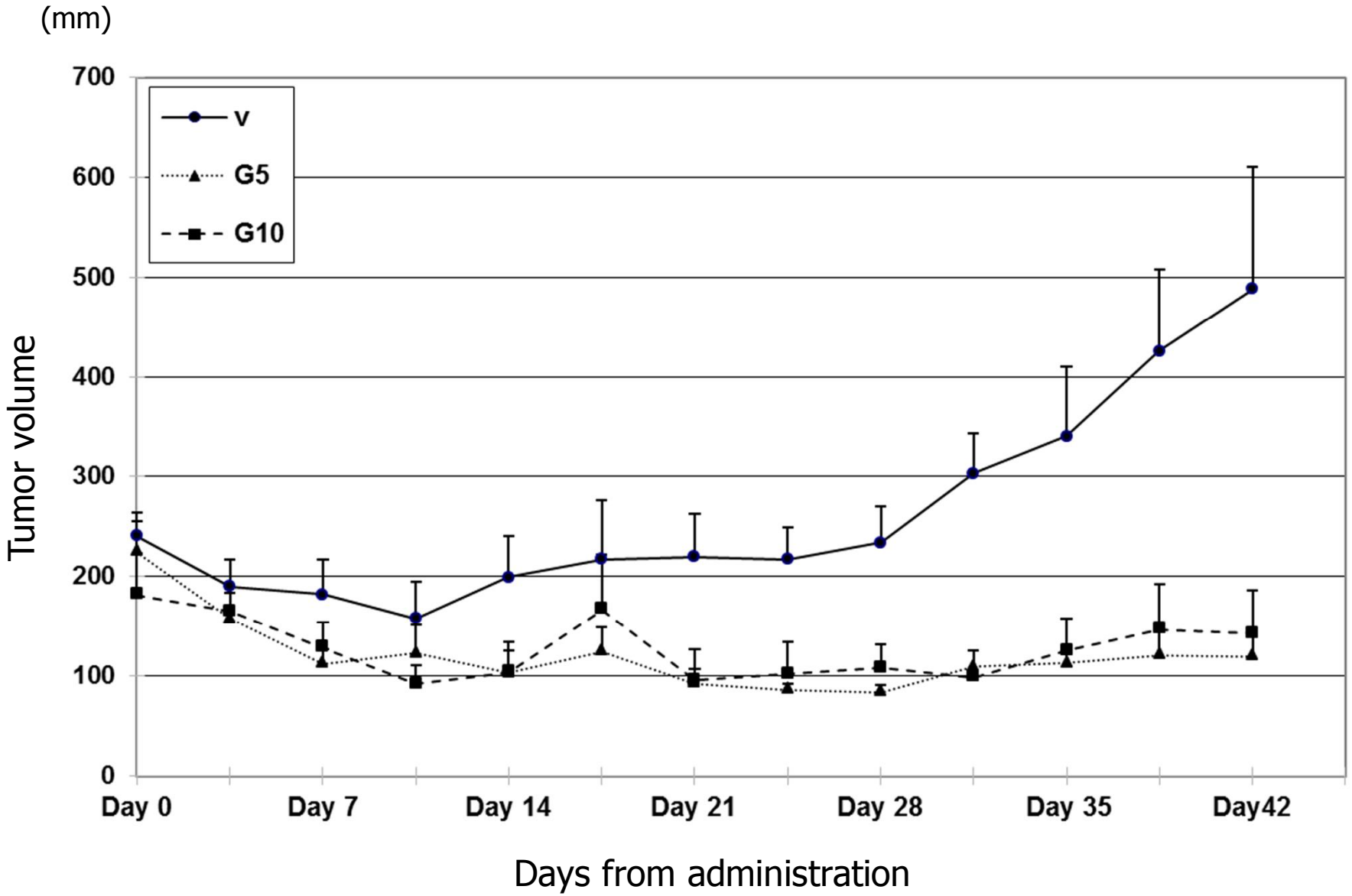

A.
PC-9
PC9-GR
pSrc
GAPDH
Relative density
Relative density
Fold change
3
2.5
2
1.5
1
0.5
0
*
0hr
1hr
3hr
6hr
12hr
24hr
0hr
1hr
3hr
6hr
12hr
24hr
B.
PC-9
PC9-GR
Gefitinib(2μM)
−
+
−
+
EGFR
pEGFR
ERK
pERK
DUSP6
/MKP3

Fig. S4


(mm)
700
600
500
400
300
200
100
0
Tumor volume
v
G5
G10
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day42
Days from administration