Effect of AZD1480 in an Epidermal Growth Factor Receptor-Driven Lung Cancer Model

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ABSTRACT

STAT3 plays a vital role in inducing and maintaining a pro-carcinogenic inflammatory microenvironment and is reported to be a critical mediator of the oncogenic effects of EGFR mutations. STAT3 activation is mediated through JAK family kinases. We investigated the effect of the JAK1/2 inhibitor AZD1480 on lung tumors induced by an activating EGFR mutation. Three EGFR tyrosine kinase inhibitor-resistant cell lines, RPC-9, PC-9/Van-R, and PC-9/ER3, were established from PC-9 harboring an EGFR exon19 deletion mutation. Their resistance mechanisms were caused mainly by a secondary mutation of T790M (RPC-9, PC-9/Van-R) or JAK2 activation (PC-9/ER3). Both resistant and parent cells were sensitive to AZD1480. AZD1480 (30 or 50 mg/kg/day, per os) reduced angiogenesis and revealed significant tumor regression in a mouse xenograft model using PC-9 cells. Subsequently, the transgenic mice expressing *Egfr* delE748-A752 were treated with AZD1480 (30 mg/kg/day) or vehicle alone. The numbers of lung tumors (long axis exceeding 1 mm) in the AZD1480-treated group and control group were 0.37 ± 0.18 and 2.25 ± 0.53 ($p<0.001$), respectively. AZD1480 treatment suppressed pSTAT3, pJAK1, and pJAK2. To evaluate the efficacy of AZD1480 on survival, AZD1480 (n=13) or vehicle (n=13) was administered orally from 7 weeks of age. The median survival time in the AZD1480-treated group (217 days) was significantly greater than that in the control group (106 days) (log-rank test, $p<0.0001$). In conclusion, AZD1480 may be effective against lung tumors driven by an activating EGFR mutation.

INTRODUCTION

Although the overall incidence of lung cancer has decreased by 0.8% annually from 1999 to 2005, it remains the leading cause of death by malignant tumors worldwide (1). Adenocarcinoma, the most prevalent histology, is present in 50% of non-small cell lung cancer (NSCLC) (2). Most lung adenocarcinoma-associated somatic epidermal growth factor receptor (EGFR) tyrosine kinase mutations were in-frame deletions in exon 19 and a point mutation (leucine to arginine [L858R]) in exon 21 (3). Patients whose tumors contain either of these mutations exhibit increased sensitivity to tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib (4).

One of the important signaling mediators downstream in human cancers is signal transducer and activator of transcription 3 (STAT3). STAT3 is also persistently activated in ~50% of NSCLC primary tumors and lung cancer-derived cell lines (5). STAT3 is activated by phosphorylation of Tyr705, leading to dimerization, nuclear translocation, DNA binding, and gene activation (6). STAT3 activation by cytokines is mediated through the Janus family kinases (JAK) or SRC (7). JAKs include four family members: JAK1, JAK2, JAK3, and TYK2. JAK1, JAK2, and TYK2 are expressed ubiquitously, whereas expression of JAK3 is primarily restricted to the lymphoid lineage (8). JAK1/2 inhibitors such as AZD1480 suppressed activation of STAT3 and showed anticancer effects using human cancer cell lines and xenograft tumors, including lung cancer (9). However, the effect of JAK1/2 inhibitors on NSCLC harboring an EGFR mutation remains unclear.

We established transgenic mice expressing the delE748-A752 mutant of mouse *Egfr*, which corresponded to the delE746-A750 mutant of human EGFR (10). In the transgenic mice, pSTAT3 was overexpressed in the bronchioloalveolar carcinoma component around the adenocarcinoma center (11). In the present study, the efficacy of AZD1480 was evaluated using EGFR mutant cell lines, xenograft mice, and transgenic mice.

MATERIALS AND METHODS

Reagents

Gefitinib and AZD1480, a 4-(pyrazol-3-ylamino) pyrimidine derivative, were kindly provided by AstraZeneca and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies. AZD1480 was suspended in water supplemented with 0.5% hypromellose and 0.1% Tween 80 for *in vivo* experiments. Rabbit antisera

against EGFR, phospho-specific EGFR (pEGFR; pY1068), phospho-specific STAT3 (pSTAT3; Tyr705), phospho-specific JAK1 (pJAK1; Tyr1022/1023), phospho-specific JAK2 (pJAK2; Tyr1007/1008), mitogen-activated protein kinase (MAPK), phospho-specific MAPK (p-MAPK; pT202/pY204), AKT, phospho-specific AKT (pAKT; pSer473), cleaved PARP, cleaved caspase-3 (Asp175; 5A1E), and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against STAT3, JAK1, and JAK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rat antisera against CD31 (PECAM-1) were purchased from Dianova (Hamburg, Germany).

Cell Lines

HEK293T cells were cultured at 37.0°C with 5% CO_2 by using Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% minimum essential medium, nonessential amino acid solution, and 2 mM L-glutamine. PC-9 is a lung adenocarcinoma cell line that has an in-frame deletion mutation at exon 19 of EGFR. PC-9 and H1975 were purchased from Immuno-Biological Laboratories (Takasaki, Gunma, Japan) and the American Type Culture Collection (Manassas, VA), respectively. A gefitinib-resistant cell line (RPC-9), a vandetanib-resistant cell line (PC-9/Van-R), and an erlotinib-resistant cell line (PC-9/ER3) were established from a parental PC-9 cell line in our laboratory (12-14). RPC-9 and PC-9/VanR cells were 400- and 138-fold more resistant to gefitinib than PC-9 cells, respectively. PC-9/ER3 cells were 136-fold more resistant than the parental cells to erlotinib. The resistance mechanism of RPC-9 and PC-9/Van-R was due mainly to a secondary mutation of T790M and that of PC-9/ER3 was due to JAK2 activation (12-14). These cell lines were cultured in RPMI-1640, 10% fetal bovine serum, and 1% penicillin/streptomycin in a tissue culture incubator at 37°C under 5% CO_2.

Transfection of EGFR

The EGFR expression vectors were constructed by inserting the full-length reverse transcription–polymerase chain reaction (RT–PCR) product of human EGFR into the pcDNA3.1-Zeo vector (Invitrogen, Carlsbad, CA). The deletion mutation was generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA). The mutation was confirmed by sequencing. The EGFR expression vector was kindly provided by Professor Takata (Radiation Biology Center, Kyoto University) and was introduced into HEK293T cells using Fugene6 (Roche Applied Science, Basel, Switzerland).

Stable EGFR-expressing clones were isolated by limiting dilution.

Cell Viability Assay

Growth inhibition was measured using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15). Briefly, the cells were plated on 96-well plates at a density of 2,000 per well and continuously exposed to each drug for 96 h. Each assay was performed in triplicate or quadruplicate. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was used to evaluate the sensitivity of gefitinib or AZD1480.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay 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 -glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate] containing a protease inhibitor tablet (Roche Applied Sciences GmbH, Basel, Switzerland). Mouse lung tissues were frozen in liquid nitrogen, crushed by applying pulsed pressure, and lysed in RIPA buffer. Each protein was separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with enhanced chemiluminescence plus reagents (GE Healthcare Biosciences, Tokyo, Japan).

Xenograft Model

Female athymic mice at 7 weeks of age were purchased from Japan Charles River Co. (Kanagawa, Japan). All mice were provided with sterilized food and water and were housed in a barrier facility under a 12-h light/dark cycle. Cells (2×10^6) were injected subcutaneously into the backs of the mice. When the average tumor volume reached ~100 mm^3, the mice were randomly assigned to one of three groups (8 to 10 mice per group) that received vehicle, or 30 or 50 mg/kg/day AZD1480. Vehicle and AZD1480 were administered once daily, six times per week by gavage. Tumor volume ($width^2 \times length/2$) was determined periodically. After completion of treatment, two mice per group were killed, and the tumor specimens were obtained for analysis. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research.

Transgenic Mouse Model

We generated transgenic mice expressing the delE748-A752 mutant of mouse *Egfr*, which is equivalent to the delE746-A750 mutant (10). The transgenic mice used the SP-C promoter to express mutated *Egfr* in type II alveolar cells. In the absence of gefitinib treatment, the transgenic mice developed adenocarcinoma at 6 to 7 weeks of age and died due to tumor progression at 13 to 18 weeks (10). Transgenic mice were randomly assigned to five groups that received either vehicle alone or AZD1480 (1, 10, 30, or 50 mg/kg/day) from 7 to 10 weeks of age. Vehicle and AZD1480 were administered once daily, six times per week by gavage. The number of superficial lung tumors (long axis exceeding 1 mm) in the left lung was counted at 10 weeks of age. To evaluate the effect of AZD1480 on survival in EGFR transgenic mice, the mice were treated with AZD1480 (30 mg/kg/day) or vehicle alone from 7 weeks of age until they died.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were cut to a thickness of 5 μm, placed on glass slides, and then deparaffinized in xylene and graded alcohol. The antigen was incubated in 10 mM sodium citrate buffer (pH 6.0) for 10 min in a 95°C water bath. The sections were then blocked for endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 10 min. The slides were rinsed with Tris-buffered saline containing 0.1% Tween 20, and the sections were blocked with normal goat serum for 60 min. The sections were incubated with anti-pSTAT3 monoclonal antibody or anti-mouse CD31 antibody overnight at 4°C. The sections were amplified using biotinylated anti-rabbit (or anti-rat) antibodies and avidin-biotinylated horseradish peroxidase conjugate for 10 min (LSABTM 2 Kit; DakoCytomation, Glostrup, Denmark), and then reacted with 3,3-diaminobenzidine. Finally, the sections were counterstained with hematoxylin. CD31 blood vessels or pSTAT3 cells were counted in six random fields (×200). All immunohistochemical analyses were completed independently by two investigators (T.M. and N.T.).

Statistical Analysis

Statistical analysis was performed using the SPSS Base System™ and Advanced Statistics™ programs (SPSS, Chicago, IL). Group differences were compared using Student's *t*-test. Survival time was defined as the period from initiation of treatment to death. Overall survival curves were calculated using the

Kaplan–Meier method. A p value less than 0.05 was deemed to indicate statistical significance.

RESULTS

Sensitivity to AZD1480 *In Vitro*

HEK293T cells transfected with the EGFR deletion mutant demonstrated increased expression of pJAK1, pJAK2, and pSTAT3, as well as pEGFR, than control cells (Fig. 1A). Next, inhibition of STAT3 using AZD1480 was examined in EGFR-addicted lung cancer cells. Figure 1B shows dose-response curves for PC-9 and RPC-9 cells using gefitinib or AZD1480. The IC_{50}s of gefitinib were 0.031 ± 0.004 μM for PC-9 cells and 6.261 ± 0.219 μM for RPC-9 cells. The IC_{50}s of AZD1480 were 2.285 ± 0.254 μM for PC-9 cells, 1.753 ± 0.253 μM for RPC-9 cells, 1.124 ± 0.059 μM for PC-9/VanR cells, and 0.985 ± 0.053 μM for PC-9/ER3 cells. Protein expression after treatment with gefitinib or AZD1480 in PC-9 cells is shown in Fig. 1C. Although 0.1 μM gefitinib inhibited pEGFR, pAKT, and pMAPK in PC-9 cells, pSTAT3 was not suppressed even in 10 μM of the drug. Although 0.001 μM AZD1480 suppressed pJAK1 and pJAK2, pSTAT3 was inhibited by 0.1 μM of the drug.

Effects of AZD1480 on PC-9 Tumors in the Xenograft Model

The tumor volume receiving vehicle, 30 mg/kg/day AZD1480, or 50 mg/kg/day AZD1480 is shown in Fig. 2A. PC-9 tumors in both AZD1480-treated groups were significantly smaller than those in the vehicle-treated group (vehicle *vs*. 30 mg/kg/day AZD1480, $p<0.05$; vehicle *vs*. 50 mg/kg/day AZD1480, $p<0.01$; day 21). We next investigated the antiangiogenic effect of AZD1480 on PC-9 tumors. Following a week of treatment with AZD1480 (50 mg/kg/day), tumor specimens were immunostained for endothelial cell marker CD31 (Fig. 2B). We found a marked reduction in CD31-positive tumor blood vessels in AZD1480-treated mice compared with control ($p<0.0001$, Fig. 2C).

The Effect of AZD1480 on Activating *EGFR*-driven Lung Cancer

Pathologically, the EGFR transgenic mice did not harbor lung tumors at 1 week and 3 weeks; however, they developed adenocarcinoma at 7 weeks (Fig. 3A). Not surprisingly, EGFR was activated even at 1 week. pSTAT3 expression was higher at 7 weeks than at 3 weeks. Figure 3B shows that gefitinib could inhibit pEGFR, but not pJAK1, pJAK2, or pSTAT3, and AZD1480 suppressed pJAK1, pJAK2, and

pSTAT3. The numbers of lung tumors (long axis exceeding 1 mm) ± standard error in the AZD1480-treated groups (30 and 50 mg/kg) and control group were 0.38 ± 0.18, 0.13 ± 0.13, and 2.25 ± 0.53 (t-test, $p<0.001$), respectively (Fig. 3C). The number of tumors observed with 1 and 10 mg/kg AZD1480 did not differ significantly from that in the control group. Pathological examination also revealed a marked reduction in tumors in the 30 and 50 mg/kg groups; however, tumors in the 1 and 10 mg/kg groups were comparable with that in the control group (Fig. 3D).

Following these findings, AZD1480 (30 mg/kg) was administrated once to transgenic mice. The lungs were removed 2, 6, 12, or 16 h after treatment. The lung specimens were immunostained using pSTAT3 antibody (Fig. 4A). The specimens collected at 2 and 6 h after AZD1480 administration showed decreased expression of pSTAT3, whereas the specimens collected at 12 and 16 h showed increased expression of pSTAT3 (Fig. 4B). Subsequently, protein was extracted from each removed lung and was subjected to Western blotting. Suppression of pSTAT3 was noted at 2 and 6 h after AZD1480 administration and cleaved PARP and caspase 3 were also observed (Fig. 4C).

To evaluate the effect of AZD1480 on survival in the EGFR transgenic mice, AZD1480 (30 mg/kg/day, n = 13) or vehicle (n = 13) was administered by gavage from 7 weeks of age. The AZD1480-treated group demonstrated a greater weight than that of the vehicle-treated group ($p<0.001$, 9 weeks) (Fig. 5A). The median survival time in the AZD1480-treated group (217 days) was significantly greater than that in the control group (106 days) (log-rank test, $p<0.0001$, Fig. 5B).

DISCUSSION

STAT3 has essential functions in both tumor cells and the tumor microenvironment to facilitate cancer progression. Multiple studies from independent groups have demonstrated evidence for STAT3 activation in nearly 50% of lung cancers (16). We found that AZD1480, which targets the JAK/STAT3 signal pathway, was effective for lung tumors harboring an exon 19 deletion mutation.

STAT3 can be activated by EGFR, whereas it has been shown that activation of STAT3 by EGFR is indirect, and also acts through JAK kinase via IL-6 (9, 16, 17). We previously showed that STAT3 was less suppressed compared with EGFR despite the administration of gefitinib to EGFR-transgenic mice: signals from other upstream factors might activate STAT3 even in EGFR-driven lung cancer (11). We also reported that increased pJAK2 expression was partially due to acquired erlotinib resistance (14).

In the present study, inducing a deletion mutation of EGFR into HEK293T cells increased expression of pJAK1, pJAK2, and pSTAT3, as well as that of pEGFR (Fig. 1A). Thus, we hypothesized that AZD1480 was effective in terms of targeting EGFR-mutated lung cancer and overcoming acquired resistance to EGFR-TKI.

AZD1480 showed IC_{50}s of approximately 1–2 μM for PC-9, RPC-9, PC-9/VanR, and PC-9/ER3 cells. Thus, no cross-resistance between EGFR-TKI and AZD1480 was found. The different AZD1480 concentrations (0.001 and 0.1 μM) inhibiting pJAK1/JAK2 and pSTAT3, respectively (Fig. 1C), suggest the existence of an upstream protein other than EGFR, JAK1, and JAK2. AZD1480 did not block tumor cell growth *in vitro* at levels that produced maximal inhibition of pSTAT3, although tumor growth was inhibited directly *in vivo* (9, 18). We also confirmed that AZD1480 was effective in the xenograft model (Fig. 2A). Constitutive activation of STAT3 within tumor cells and stromal cells promotes cancer cell proliferation, invasion, angiogenesis, and immune evasion (19). The effect of AZD1480 on the tumor microenvironment was recently demonstrated (18). In the present study, AZD1480 prevented angiogenesis (Fig. 2B, 2C), confirming those results.

Next, transgenic mice expressing the delE748-A752 mutant of mouse *Egfr* were treated with AZD1480. In contrast to the case *in vitro* (Fig. 1C), AZD1480 suppressed expression of pSTAT3 more than that of pJAK1 and pJAK2 (Fig. 3B). AZD1480 (30 and 50 mg/kg) by gavage reduced lung tumors in transgenic mice (Fig. 3C). In this EGFR-addicted lung cancer model, the JAK/STAT3 pathway is not the main route of carcinogenesis or cancer progression; however, interestingly, inhibition of JAK/STAT3 signals leads to significant tumor reduction without suppressing pEGFR (Fig. 3B). In addition, pSTAT3 was reduced temporally, and apoptosis occurred in the transgenic mice (Fig. 4). To confirm whether this tumor regression is specific to mice containing the EGFR deletion mutation, L858R transgenic mice (20) were used. AZD1480 (30 and 50 mg/kg) decreased the number of superficial lung tumors along with the reduction in pSTAT3 (Supplemental Fig. 1). Thus, AZD1480 was effective for the EGFR-addicted lung cancer model in terms of tumor suppression.

Finally, the effect of AZD1480 on survival was demonstrated (Fig. 5B). The median survival time in the AZD1480-treated group (217 days) was almost twice that in the control group (106 days). Transgenic mice treated with AZD1480 survived for a period of time similar to those treated with gefitinib (10). The prolongation of survival by AZD1480 in-EGFR transgenic mice compared with vehicle treatment

was to our knowledge confirmed here for the first time. AZD1480 may be effective for treatment of lung tumors induced by an activating EGFR mutation. The lack of cross-resistance between EGFR-TKI and AZD1480, suggests its usefulness for EGFR-TKI-resistant lung cancer. The role of AZD1480 in the treatment of EGFR addicted lung cancer should be clarified in further clinical studies.

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FIGURE LEGENDS

Figure 1. Protein expression and sensitivity to AZD1480 *in vitro*.

(A) HEK293T cells transfected with the EGFR deletion mutant displayed increased expression of pJAK1, pJAK2, and pSTAT3, as well as pEGFR compared with control cells. (B) Dose-response curves for PC-9 and RPC-9 cells using gefitinib or AZD1480 are shown. The IC_{50}s of gefitinib were 0.031 ± 0.004 μM for PC-9 and 6.261 ± 0.219 μM for RPC-9. The IC_{50}s of AZD1480 were 2.285 ± 0.254 μM for PC-9 and 1.753 ± 0.253 μM for RPC-9. (C) Protein expression after treatment with gefitinib or AZD1480 in PC-9 cells is shown. Although 0.1 μM gefitinib inhibited pEGFR, pAKT, and pMAPK in PC-9 cells, pSTAT3 was not suppressed even upon administration of 10 μM of the drug. Although 0.001 μM AZD1480 suppressed pJAK1 and pJAK2, pSTAT3 was inhibited by 0.1 μM.

Figure 2. Effects of AZD1480 on PC-9 xenograft tumors.

(A) The tumor volume receiving vehicle, 30 mg/kg/day AZD1480 or 50 mg/kg/day AZD1480 is shown. Both AZD1480-treated groups had significantly smaller PC-9 tumors than the vehicle-treated group (vehicle *vs*. 30 mg/kg/day AZD1480: $p<0.05$; vehicle *vs*. 50 mg/kg/day AZD1480: $p<0.01$; day 21). (B) Following a week of treatment with AZD1480 (50 mg/kg/day), tumor specimens were immunostained for the endothelial cell marker CD31. (C) A more than threefold reduction in CD31 tumor blood vessels in AZD1480-treated mice compared with control mice was observed ($p<0.0001$).

Figure 3. The effect of AZD1480 on EGFR transgenic mice.

(A) A pathological specimen shows that EGFR transgenic mice did not harbor lung tumors at 1 and 3 weeks, but they developed adenocarcinoma at 7 weeks. Although EGFR was activated even at 1 week, pSTAT3 showed an increased expression at 7 weeks compared with 3 weeks. (B) Gefitinib inhibited pEGFR but not pJAK1, pJAK2, or pSTAT3. Meanwhile, AZD1480 suppressed pJAK1, pJAK2, and pSTAT3. (C) The numbers of lung tumors (long axis exceeding 1 mm) ± standard error in the AZD1480-treated groups (30 and 50 mg/kg) and control group were 0.38 ± 0.18, 0.13 ± 0.13, and 2.25 ± 0.53 (*t*-test, $p<0.001$), respectively. The number of tumors observed with 1 and 10 mg/kg AZD1480 treatment did not differ significantly from that in the control group. (D) Pathological examination revealed marked reduction of the tumor in the 30 and 50 mg/kg groups; however, tumors in the 1 and 10 mg/kg

groups were comparable with that of the control.

Figure 4. Protein expression following short-term exposure to AZD1480.

(A) AZD1480 (30 mg/kg) was administered once to the transgenic mice. The lungs were removed 2, 6, 12, or 16 h after AZD1480 treatment. Lung specimens were then immunostained using a pSTAT3 antibody. The specimens collected at 2 and 6 h after AZD1480 administration showed decreased expression of pSTAT3. The specimens collected at 12 and 16 h showed increased expression of pSTAT3. (B) Graphical presentation of pSTAT3 expression. (C) Protein was extracted from each removed lung and subjected to Western blotting. Suppression of pSTAT3 was noted at 2 and 6 h after AZD1480 administration; cleaved PARP and caspase 3 were also observed.

Figure 5. Long-term treatment of AZD1480 in EGFR transgenic mice.

(A) AZD1480 (30 mg/kg/day, n = 13) or vehicle (n = 13) was administered orally from 7 weeks of age. The AZD1480-treated group displayed a higher weight than the vehicle-treated group (p<0.001 at 9 weeks). (B) Survival curves. The median survival time in the AZD1480-treated group (217 days) was significantly greater than that in the control group (106 days) (log-rank test, $p<0.0001$).

Supplemental Figure 1. AZD1480 (30 and 50 mg/kg) decreased the number of superficial lung tumors and reduced pSTAT3 expression in L858R-transgenic mice.

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Figure 1

A

B   CD31

C
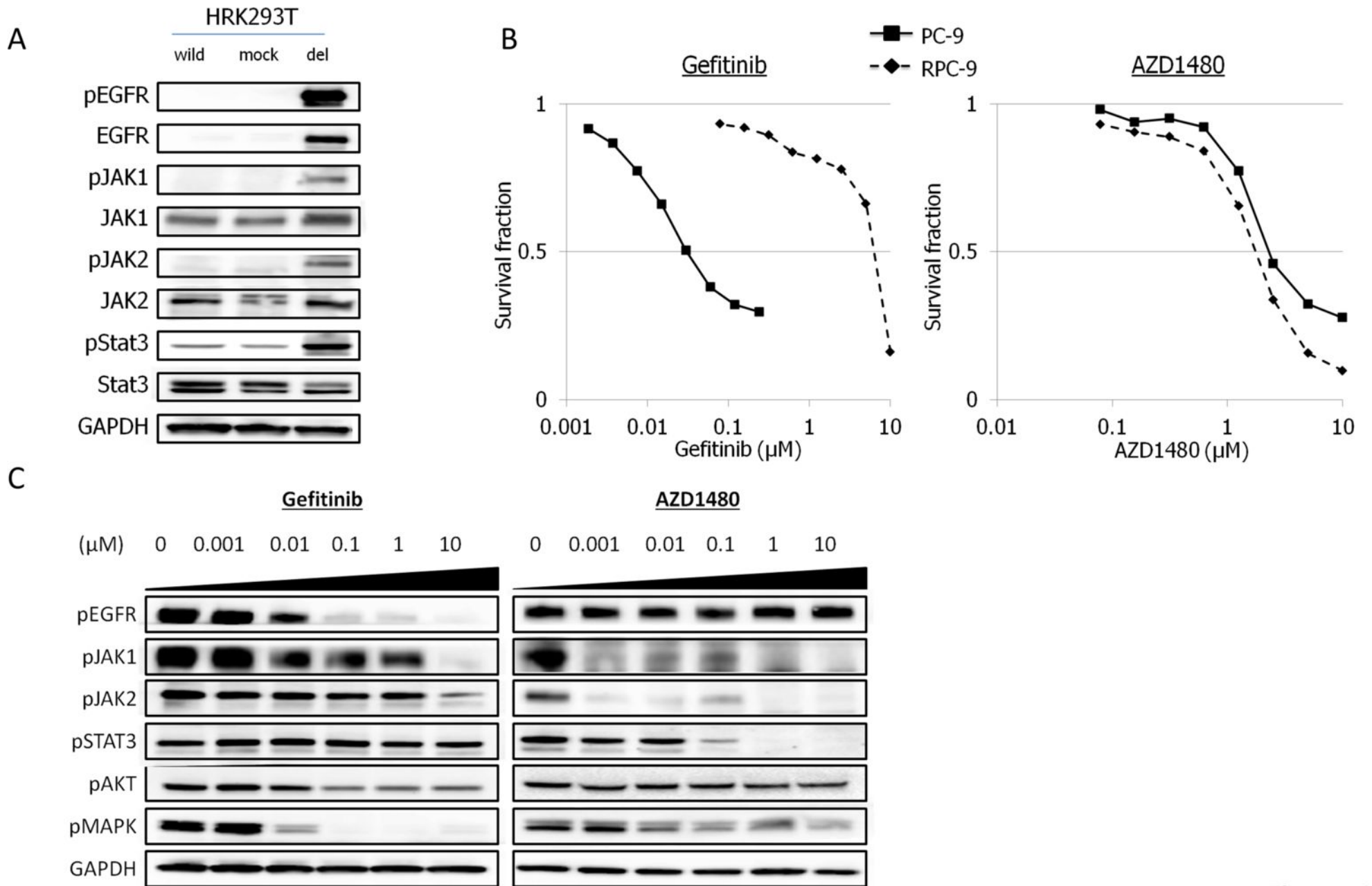
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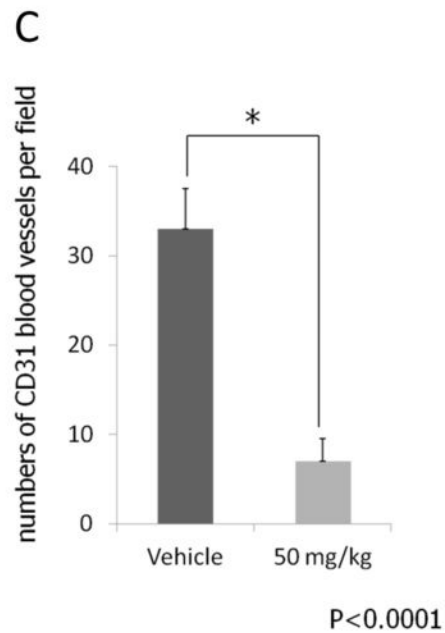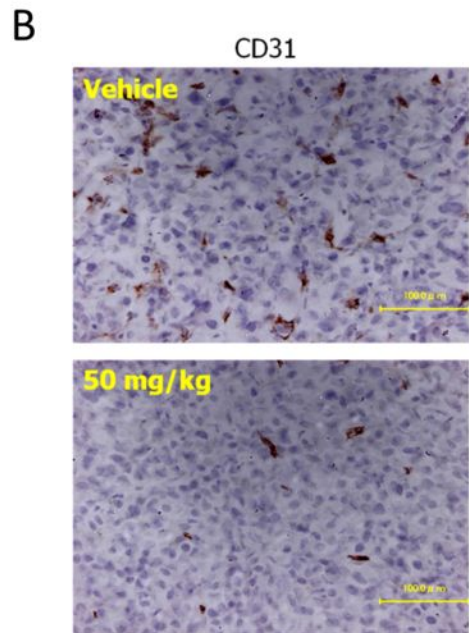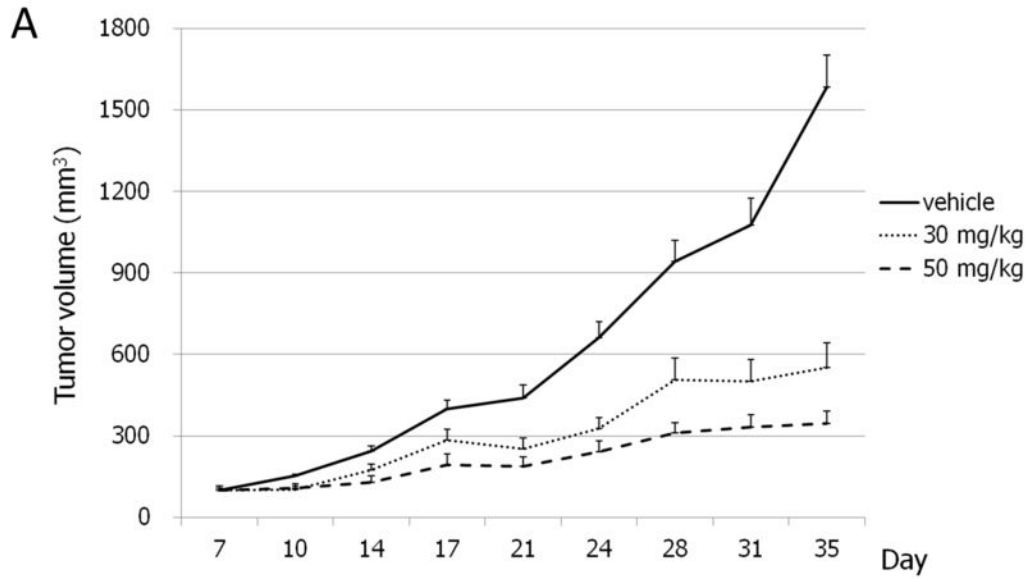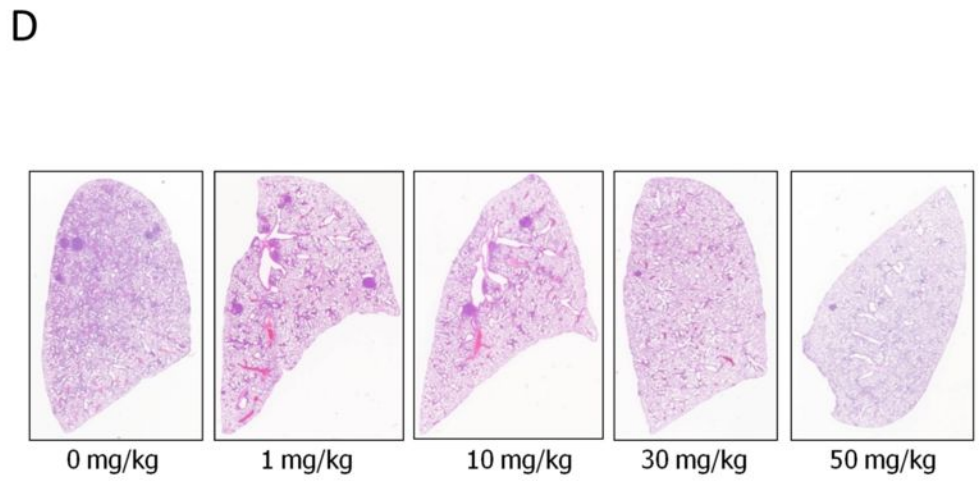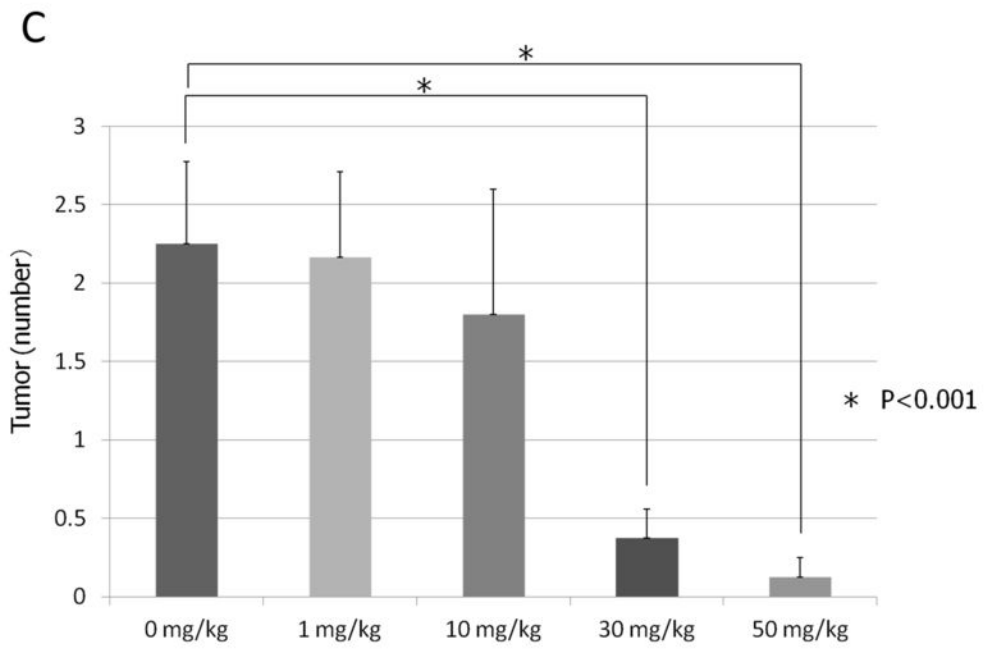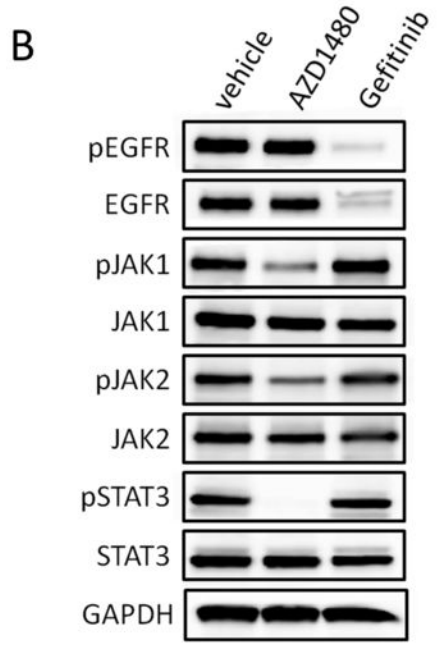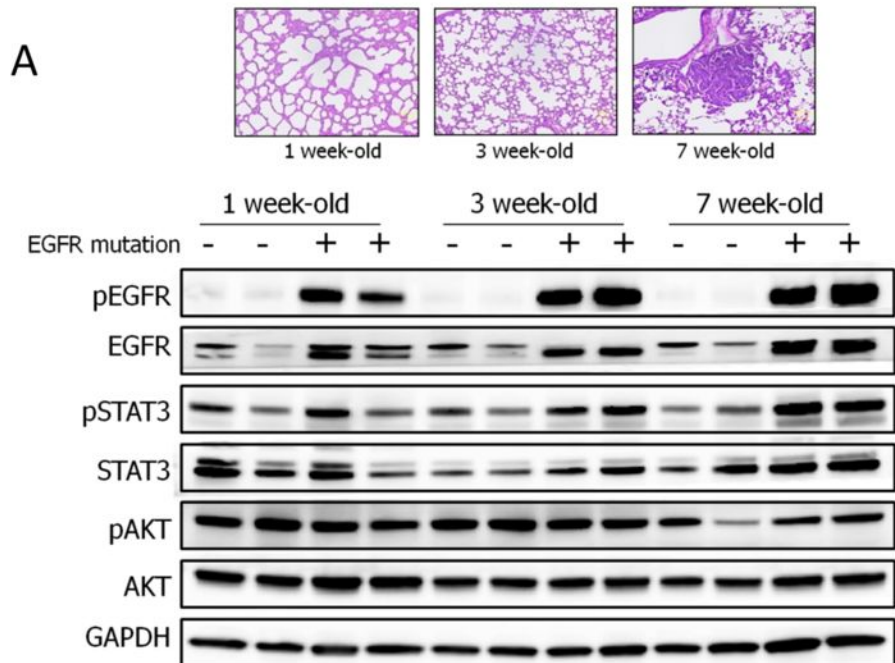
Figure 2

Figure 3

Figure 4

Figure 5