

Effect of Vandetanib on Lung Tumorigenesis in Transgenic Mice Carrying an Activating *Egfr* Gene Mutation

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Vandetanib (ZactimaTM) is a novel, orally available inhibitor of both vascular endothelial growth factor receptor-2 (VEGFR-2) and epidermal growth factor receptor (EGFR) tyrosine kinase. In the present study, a line of transgenic mice with a mouse *Egfr* gene mutation (delE748-A752) corresponding to a human *EGFR* mutation (delE746-A750) was established. The transgenic mice developed atypical adenomatous hyperplasia to adenocarcinoma of the lung at around 5 weeks of age and died of lung tumors at approximately 17 weeks of age. In the mice treated with vandetanib (6 mg/kg/day), these lung tumors disappeared and the phosphorylations of EGFR and VEGFR-2 were reduced in lung tissues to levels comparable to those of non-transgenic control mice. The median overall survival time of the transgenic mice was 28 weeks in the vandetanib-treated group and 17 weeks in the vehicle-treated group. Vandetanib significantly prolonged the survival of the transgenic mice (log-rank test, $p < 0.01$); resistance to vandetanib occurred at 20 weeks of age and the animals died from their lung tumors at about 28 weeks of age. These data suggest that vandetanib could suppress the progression of tumors harboring an activating EGFR mutation.

Key words: vandetanib, VEGFR, EGFR, nonsmall cell lung cancer, transgenic mouse

For the year 2012, the WHO reported 1.59 million deaths from lung cancer [<http://www.who.int/mediacentre/factsheets/fs297/en/> (accessed Dec 1, 2015)], making it the most common cause of cancer death globally. Despite progress in the treatment of lung cancer by surgical resection, radiotherapy, and chemotherapy, the prognosis of patients with advanced disease remains poor. Indeed, the 5-year survival rate for patients with advanced non-small-cell lung cancer (NSCLC) is around 15% [1].

The growth and maintenance of tumors such as NSCLC is dependent upon cellular as well as extracellular processes. Epidermal growth factor receptor (EGFR) signaling regulates many of the cellular processes essential for tumor initiation and progression, including motility, adhesion, tumor invasion, cell survival, angiogenesis, and proliferation [2]. The importance of EGFR in cancer has been therapeutically exploited by the use of agents such as EGFR tyrosine kinase inhibitor (TKI) and EGFR monoclonal antibodies, both of which have been proven to be of

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clinical benefit in the treatment of advanced NSCLC [3, 4].

The expansion, invasion, and metastasis of malignant tumors are processes that depend upon an adequate supply of nutrients and oxygen from functional blood vessels. Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) play important roles in the formation of new blood vessels (angiogenesis) [5]. VEGFR signaling regulates vascular permeability and is crucial to vasculogenesis, angiogenesis, and endothelial integrity and survival [6, 7]. In most types of cancers, including NSCLC, breast cancers, prostate cancer, and colorectal cancers, VEGF is overexpressed and is generally associated with increases in microvascular density [7, 8]. VEGF and VEGFR are therefore attractive targets for anticancer therapy. Several studies have shown an improvement in the outcomes of patients with metastatic NSCLC, colorectal cancer, and breast cancer who were treated with a combination of bevacizumab, an anti-VEGF antibody, and chemotherapy [9–13]. Moreover, several approaches to the blocking of VEGF activity are under development, including anti-VEGF antibodies, anti-VEGFR antibodies, VEGF trapping, and VEGFR TKI [8].

Lung cancer development and progression likely involve the dysregulation of both EGFR and VEGFR signaling. Accordingly, the inhibition of these two receptor-signaling pathways is an attractive strategy to treat advanced NSCLC [11–13]. Vandetanib is an orally available inhibitor of VEGFR-2, EGFR, and RET tyrosine kinase activity [14]. A randomized, double-blind Phase III study (ZEPHYR) did not demonstrate an overall survival benefit for vandetanib versus placebo, but vandetanib did show statistically significant advantages versus placebo in terms of the progression-free survival and objective response rate [15]. Subsequently, 5 randomized clinical trials were performed to compare chemotherapy with or without vandetanib in patients with advanced NSCLC. The results showed a significantly longer progression-free survival by chemotherapy with vandetanib but did not show any advantage of this therapy for overall survival (OS) [16–19].

In the present study, a line of transgenic mice harboring the mouse *Egfr* mutation (delE748–A752) under the control of a type-II-pneumocyte-specific surfactant protein C (SPC) promoter [18] was estab-

lished. These transgenic mice developed a spectrum of pathological changes in their lungs, ranging from atypical adenomatous hyperplasia to invasive adenocarcinoma, and died due to multifocal invasive adenocarcinoma at around 17 weeks of age. This mouse model of lung cancer resembles the clinical course of human adenocarcinomas harboring an activating *EGFR* mutation and thus enables evaluation of the direct and indirect effects of tyrosine kinase inhibition on tumor cells and neovascular vessels, respectively, under physiologically and pathologically relevant conditions.

The specific aims of this study were to evaluate the efficacy of vandetanib in the mutated *Egfr* transgenic mouse model and to elucidate the mechanisms of acquired resistance to vandetanib.

Materials and Methods

Transgenic mice. Transgenic mice were established as reported previously [20]. These mice harbor the mouse *Egfr* mutation del E748–A752, which corresponds to the major activating human *EGFR* mutation (del E746–A750), under the control of a type-II-pneumocyte-specific SP-C promoter (kindly provided by Dr. Jeffrey A. Whitsett, the Division of Neonatology Perinatal and Pulmonary Biology at Cincinnati Children's Hospital Medical Center, OH, USA). Pathological changes ranging from atypical adenocarcinoma to invasive adenocarcinoma of the lung developed in the transgenic mice beginning at around 5 weeks of age, and the animals died from multifocal adenocarcinoma of the lung at about 17 weeks of age.

Animal husbandry. All animals were kept under pathogen-free conditions with abundant food and water, as specified in the guidelines of the Department of Animal Resources, Okayama University Advanced Science Research Center. Vandetanib (6 mg/kg; AstraZeneca, Macclesfield, UK) was given as an oral suspension once a day by gavage. The vandetanib suspension was obtained by homogenization in 1% polysorbate 80 followed by ball-milling with glass beads for 24 h.

Histology, immunohistochemistry, and immunofluorescence. The animals were killed by cervical dislocation and their lungs excised. The left lung was flash-frozen in liquid nitrogen for molecular analysis, and the right lung was inflated with 10% paraformaldehyde in phosphate-buffered saline (PBS). The

lungs were subsequently fixed in 10% paraformaldehyde overnight at room temperature, embedded in paraffin, and sectioned in 5- μ m slices (West Japan Pathology, Okayama, Japan). Heat-induced antigen retrieval was performed by treatment with a PASCAL pressure cooker (Dako, Glostrup, Denmark) according to the manufacturer's protocol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 10 min. After incubation with blocking solution for 60 min, the sections were incubated with primary antibody (1 : 50 dilution) at 4°C for 12 h, followed by a 20-min incubation with a labeled polymer horseradish peroxidase (HRP) anti-rabbit antibody (EnVision + System; Dako). The sections were then stained with diaminobenzidine chromogen and counterstained with Mayer's hematoxylin. The primary antibodies used were rabbit polyclonal anti-EGFR anti-phospho-VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-EGFR (Cell Signaling Technology, Danvers, MA, USA), and VEGFR2 (Biosource International, Camarillo, CA, USA). The effect of vandetanib on apoptosis and microvessel density was evaluated by immunofluorescence microscopy of frozen lung tissue from vandetanib-treated and vehicle-treated transgenic mice. The samples were sectioned at 8 μ m and mounted on MAS-coated glass slides (SUPERFROST; Matsunami, Osaka, Japan). These were fixed with 4% paraformaldehyde, washed with PBS, incubated with 10% bovine serum albumin in PBS for 15 min, and then incubated with a 1:100 dilution of rabbit FITC-conjugated anti-active caspase-3 polyclonal antibody (BD Biosciences, Tokyo, Japan) overnight at 4°C in the dark. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Roche Applied Sciences GmbH, Mannheim, Germany) for 2 h in the dark. After washing with PBS, the samples were incubated with a 1:100 dilution of rabbit PE-conjugated anti-CD31 polyclonal antibody (BD Biosciences) overnight at 4°C in the dark and then examined by fluorescence microscopy (LSM510; Carl Zeiss, Thornwood, NY, USA).

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Samples were extracted from whole lung tissues. RNA samples were prepared for RT-PCR using the RNeasy mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The cDNA was synthesized using

SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To specifically amplify the mutant version of mouse *Egfr*, the primer EGFR F8del (5'-CCTATTCATGCGAAGACGT-3'), which spanned the deleted portion of the *Egfr* cDNA, was designed and used with EGFR R5 (5'-AAAGTTGGAGAGTCTGTAGGGCT-3'). *Gapdh* served as the control gene and was amplified using the primers GAPDH F (5'-CGTAGACAAAATGGTGAAGG-3') and GAPDH R (5'-GTTGTCATGGATGACCTTGG-3').

Quantitative PCR. DNA was extracted from whole lung tissues using a QIAamp DNA mini kit (Qiagen). The relative level of *Egfr* expression level, as well as the *Egfr* copy number, and the *cMet* copy number were analyzed using Duplex TaqMan real-time PCR together with an ABI PRISM 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The *Egfr* primers were 5'-CTGCCAAA GTTCCAAGATGAGG-3' and 5'-GGGGCACTTCTTCACACAGG-3'; those for *cMet* were 5'-TTCCCTGTATCCACCTATCAAAGTC-3' and 5'-GGACACAGTATTTGCTATGAAGTCCG-3'. Both probes (5'-AGACACCTGCCCACCACTCATGCT-3' and 5'-ACAGACAGACTGCTATGCCTGCCCT-3', respectively) were labeled with the reporter dye 6-carboxyfluorescein (FAM). *Gapdh* was co-amplified in the same reaction mixture as an endogenous reference gene using TaqMan GAPDH control reagents (Sigma-Aldrich, Tokyo, Japan). The average expression level and copy number of *Egfr* were determined from differences in the threshold amplification cycles between *Egfr* and *Gapdh*. A similar method was used to determine the copy number of *cMet*.

Sequencing of *Egfr*. To sequence *Egfr*, DNA was extracted from whole lung tissues using a QIAamp DNA mini kit (Qiagen), and the exons encoding the intracellular domain (exons 18-22) were amplified by PCR. Primer sequences and amplification conditions were as described previously [20]. PCR products were processed with a Big Dye terminator cycle sequencing kit (Biosystems) and analyzed for the presence of mutations in the sense and antisense directions on an ABI 3100 sequencer (Biosystems).

Immunoblotting. Protein extracts were prepared from crushed whole lung tissues that had been incubated in lysis buffer (1% Triton X-100, 0.1% SDS, 50mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 10mM β -glycerol phosphate,

10 mM NaF, and 1 mM Na-orthovanadate) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland). The extracts were centrifuged at 14,500 rpm for 20 min at 4°C, and the amount of protein was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Approximately 50 µg of protein per sample was separated by SDS-PAGE using 5–15% precast gels (Bio-Rad). The separated proteins were transferred onto nitrocellulose membranes, and specific proteins were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using the following antibodies: phospho-EGFR Y1068, total EGFR, total VEGFR-2, phospho-Akt Ser473, total Akt, phospho-44/42 MAP kinase, total MAP kinase, phospho-Stat3 (Tyr705), total Stat3, β -actin, PTEN, Bcl-xL, IRS-1, phospho-IGF-1R β Y1135/1136 Y1150/1151, total IGF-1R β (1:1000 dilution; Cell Signaling Technology), phospho-VEGFR2 Y1054 (1:1000 dilution; Biosource International) and BIM (1:200 dilution; Santa Cruz Biotechnology). The secondary antibodies were anti-rabbit IgG antibodies (HRP-linked, No. 7074, species-specific whole antibodies; GE Healthcare), each of which was used at a 1:5000 dilution.

Statistical analysis. Survival time was defined as the period from birth to death. Survival curves were calculated using the Kaplan–Meier method. The log-rank test was used to detect differences in survival. Statistical significance was defined as $p < 0.05$. All statistical analyses were carried out using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Histology of the lungs of the transgenic mice.

Atypical adenomatous hyperplasia, diffuse bronchioalveolar carcinoma, and invasive adenocarcinoma of the lung were identified in the transgenic mice at 15 weeks of age (Fig. 1). The tumors overexpressed total EGFR, phosphorylated EGFR (pEGFR), VEGFR2, and phosphorylated VEGFR2 (pVEGFR2) (Fig. 2A). As shown in Fig. 2, both pEGFR and pVEGFR-2 were markedly overexpressed in the lungs of transgenic mice, suggesting that VEGFR-2 signaling contributes to tumor progression and growth in this transgenic mouse model. Ultimately, the transgenic mice died at around 17 weeks of age due to multifocal

invasive adenocarcinoma of the lung. Multiple macroscopic tumor nodules were observed on the lung surface, and no tumors were found in any other organs. In contrast, non-transgenic control mice did not develop lung tumors and survived for over 1 year.

Inhibitory effect of vandetanib on lung adenocarcinoma induced by an activating Egfr mutation in vivo. To investigate the antitumor activity of vandetanib in tumors harboring the activating *Egfr* mutation, 7-week-old transgenic mice were administered vandetanib (6 mg/kg) or vehicle daily for 7 days, after which they were killed. After administering vandetanib for 2 days, the area occupied by tumor cells decreased compared with the pre-administration value (Fig. 1B, C). Furthermore, tumor cell disappeared in the seeing length after the administration of vandetanib for 7 days (Fig. 1D).

The expression of total EGFR and total VEGFR2 in the lungs of the transgenic mice, as assessed by immunohistochemistry, was slightly suppressed in the vandetanib-treated animals compared to the animals treated with vehicle alone, while the pEGFR and pVEGFR levels were markedly decreased in the transgenic mice treated with vandetanib relative to those treated with vehicle (Fig. 2).

The effect of vandetanib on apoptosis in the lung tumors and blood vessels of the transgenic mice was also evaluated. Immunofluorescence showed increased

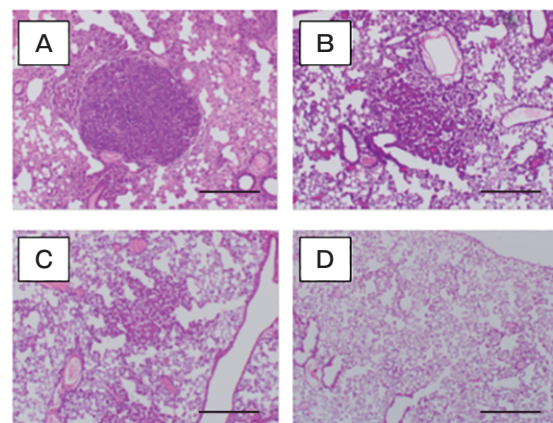


Fig. 1 A, Lung tissue of a transgenic mouse at 15 weeks of age showing diffuse and solid patterns of bronchioalveolar carcinoma; B, Adenocarcinoma of the lung in a transgenic mouse at 7 weeks of age, before vandetanib treatment; C and D, Lung tissue of transgenic mice on days 2 (C) and 7 (D) of vandetanib treatment (6 mg/kg/day). Adenocarcinoma of the lung was not apparent by day 7 of treatment. Scale bars: 100 µm

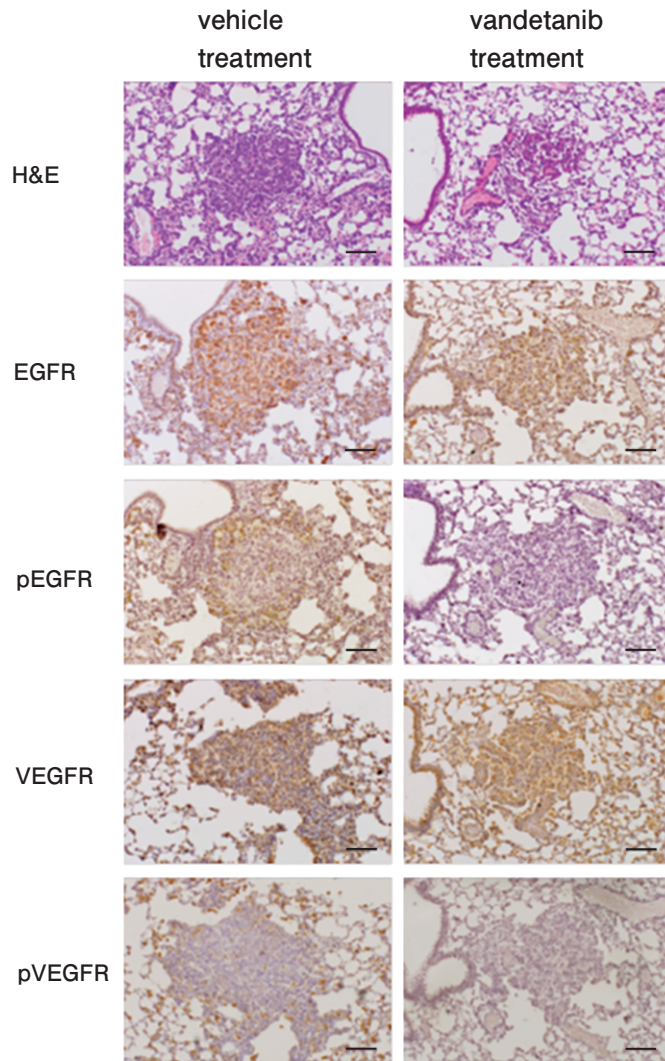


Fig. 2 Staining for total EGFR, pEGFR, total VEGFR2, and pVEGFR in lung tissues of transgenic mice at 13 weeks of age after 7 days of treatment with vandetanib (6 mg/kg/day) or vehicle. Scale bars: 100 μ m

activated caspase-3 and decreased microvessel density in the lung tumors of vandetanib-treated mice compared to vehicle-treated transgenic mice (Fig. 3A). Moreover, apoptosis was induced by vandetanib not only in tumor cells but also in CD31-positive endothelial cells (Fig. 3A). 7-week-old transgenic mice were treated with vandetanib for 7 days, after which they were killed and the proteins of the lung tissues were extracted. Total EGFR and VEGFR2 levels in the lungs of the transgenic mice were similar in the vandetanib-treated and vehicle-treated groups, while pEGFR and pVEGFR were markedly decreased in the lungs of the mice after 7 days of vandetanib treatment (Fig. 3B).

Effect of vandetanib on survival in the trans-

genic mice. Since lung adenocarcinoma in the transgenic mice regressed markedly after vandetanib treatment, the effect of vandetanib on survival was examined. Transgenic mice received vandetanib (6 mg/kg weekly on days 1–5) or vehicle orally 5 days per week from 7 weeks of age until death ($n = 6$ mice per group), (Fig. 4A). Vandetanib treatment significantly prolonged survival (log-rank test, $p < 0.01$; Fig. 4B); the median survival time of the vandetanib-treated mice was 28 weeks, whereas that of the vehicle-treated mice was 17 weeks. Multiple recurrent lung adenocarcinomas were found in the dead transgenic mice, but metastatic tumors were not detected in any other organs (data not shown). The vandetanib-treated transgenic mice were killed at various time

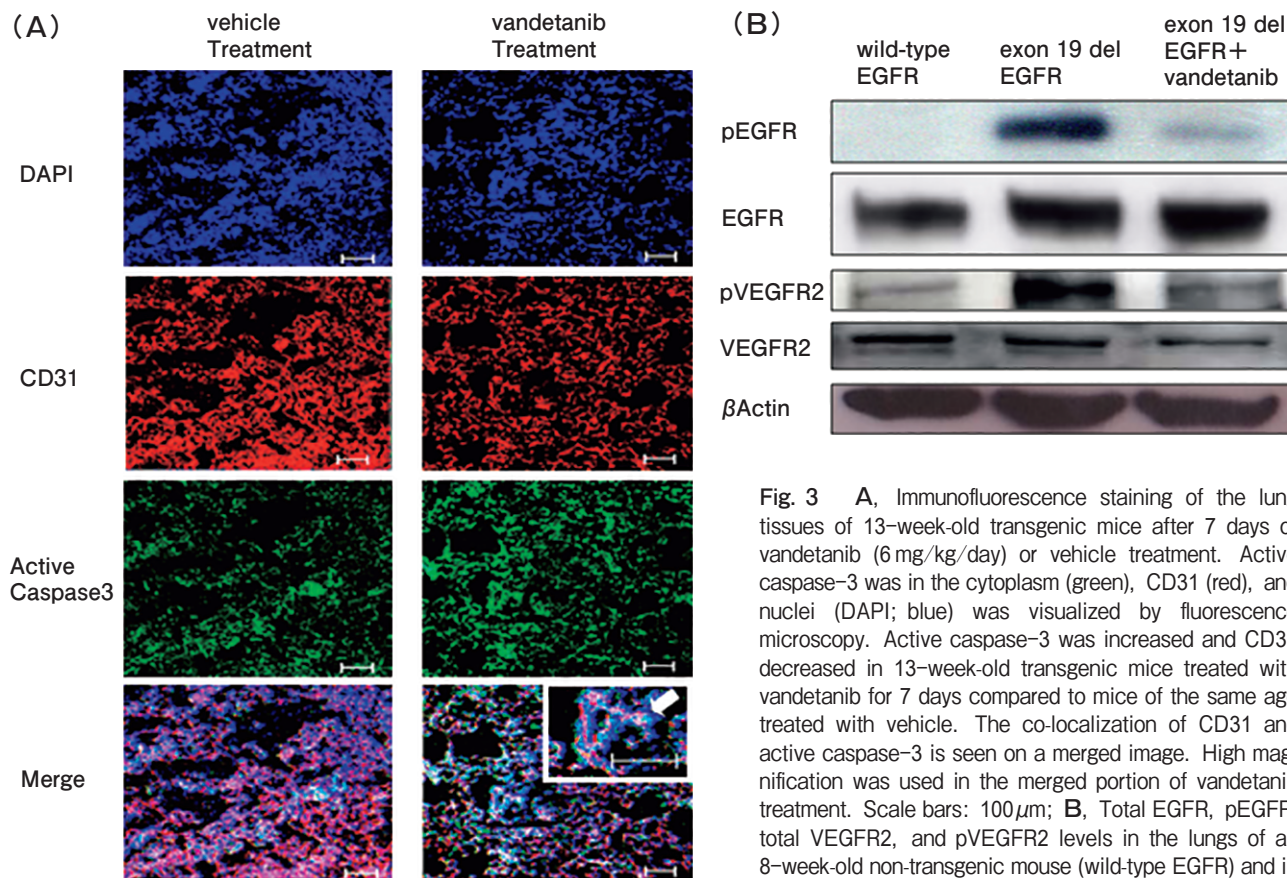


Fig. 3 **A**, Immunofluorescence staining of the lung tissues of 13-week-old transgenic mice after 7 days of vandetanib (6 mg/kg/day) or vehicle treatment. Active caspase-3 was in the cytoplasm (green), CD31 (red), and nuclei (DAPI; blue) was visualized by fluorescence microscopy. Active caspase-3 was increased and CD31 decreased in 13-week-old transgenic mice treated with vandetanib for 7 days compared to mice of the same age treated with vehicle. The co-localization of CD31 and active caspase-3 is seen on a merged image. High magnification was used in the merged portion of vandetanib treatment. Scale bars: 100 μ m; **B**, Total EGFR, pEGFR, total VEGFR2, and pVEGFR2 levels in the lungs of an 8-week-old non-transgenic mouse (wild-type EGFR) and in a transgenic mouse with adenocarcinoma of the lung. The mice were killed at 8 weeks of age, after being treated for 1 week with vandetanib (6 mg/kg/day) or vehicle. pEGFR and pVEGFR in total lysates were detected using a specific antibody against pEGFR Y1068 and pVEGFR2 Y1054, respectively.

points to assess the dynamics of tumor recurrence. Multiple recurrent adenocarcinomas were present in these mice at 20 weeks of age (Fig. 4C).

Mechanisms of acquired resistance to vandetanib. Known mechanisms of acquired resistance to EGFR-TKIs include secondary mutation of *EGFR* [21–23], *cMET* amplification [24], type 1 insulin-like growth factor receptor (IGF-1R) activation [25, 26], and PTEN inactivation [27]. Therefore, to gain insight into the mechanisms of vandetanib resistance, total and phosphorylated EGFR and VEGFR2 were examined in lung tumors from 8-week-old non-transgenic mice (wild-type *Egfr*) and 8-week-old vehicle-treated transgenic mice, as well as 8- and 20-week-old vandetanib-treated transgenic mice (1 week and 13

weeks of vandetanib treatment, which was 6 mg/kg weekly on days 1–5, respectively; Fig. 5A). Vandetanib treatment was found to inhibit both EGFR and VEGFR signaling even after the tumors had acquired resistance to vandetanib (Fig. 5A). Moreover, neither total EGFR nor VEGFR2 was overexpressed in the vandetanib-treated 20-week-old transgenic mice (Fig. 5A). Previous studies have shown that the secondary EGFR mutation T790M is the major cause of acquired resistance to gefitinib and erlotinib *in vivo* and *in vitro* [21–23]. Here, examination of the transgenic mouse tumors for genetic alterations, including T790M, based on sequencing assays of *Egfr* at exons 18–22 did not reveal the mutation within exon 20. Recently, D761Y or L747S mutations at exon 19 were shown to

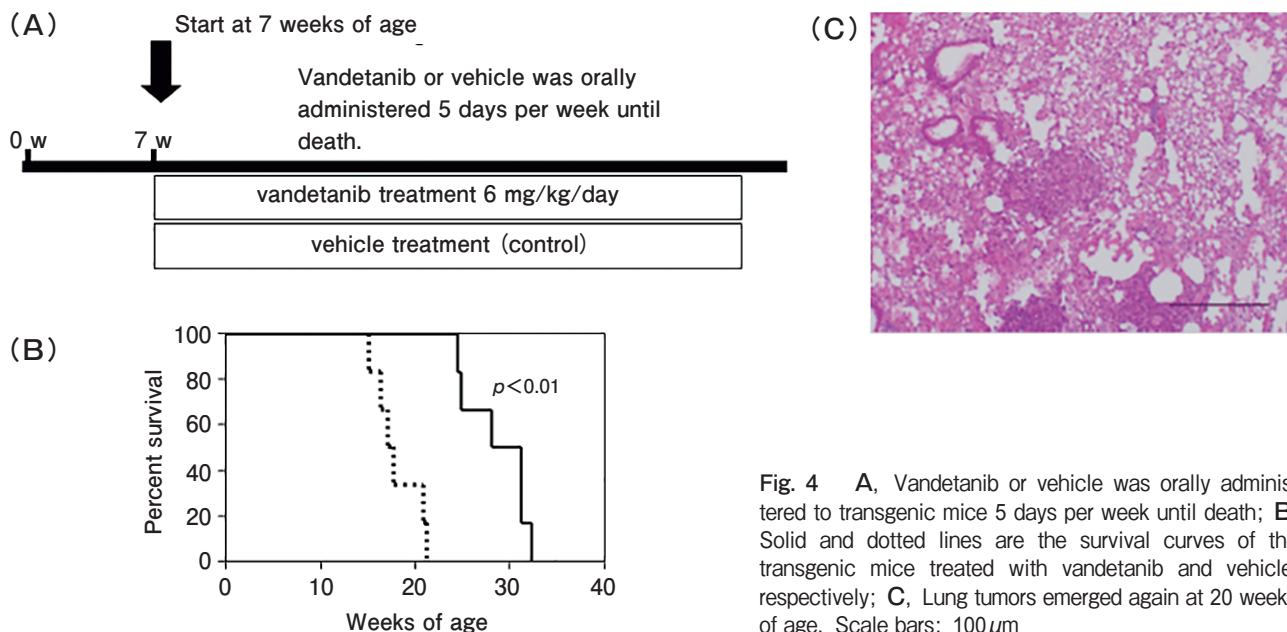


Fig. 4 A, Vandetanib or vehicle was orally administered to transgenic mice 5 days per week until death; B, Solid and dotted lines are the survival curves of the transgenic mice treated with vandetanib and vehicle, respectively; C, Lung tumors emerged again at 20 weeks of age. Scale bars: 100 μm

be responsible for acquired EGFR-TKI resistance [22, 23]; however, neither of these mutations was detected. Moreover, a comparison between the lungs of 8-week-old vehicle-treated animals and those of 20-week-old vandetanib-treated animals failed to reveal evidence of a *cMet* gene amplification (using a quantitative PCR method) during treatment with vandetanib (Fig. 5B) or altered PTEN protein expression (Fig. 5A).

Phosphorylated Akt (pAkt) levels were reduced in 8- and 20-week-old vandetanib-treated mice, whereas pSTAT3 levels were similar. In contrast, pMAPK levels were higher in 8-week-old vehicle-treated transgenic mice and 20-week-old vandetanib-treated transgenic mice than in 8-week-old vandetanib-treated transgenic mice (Fig. 5A). Note that pIGF-1R was overexpressed, although total IGF-1R remained unchanged (Fig. 5A). Thus, the mechanisms of acquired resistance to vandetanib in the transgenic mice may be associated with reestablished signaling via the insulin receptor substrate-1 (IRS-1) to the Ras kinase signaling cascade.

Vandetanib induced apoptosis in the lung tumors of the transgenic mice (Fig. 3A). In previous studies, the EGFR-ERK signaling cascade was shown to be associated with the pro-apoptotic BH3-only protein BIM in apoptosis [28-31], and BIM has been reported

to mediate EGFR-TKI-induced apoptosis in lung tumors harboring activating *EGFR* mutations [28, 30, 31]. We therefore examined the expression levels of BIM and anti-apoptotic protein Bcl-xL (basal cell lymphoma-extra large) in 8-week-old non-transgenic mice, 8-week-old transgenic mice treated with vehicle or vandetanib for 1 week, and 20-week-old transgenic mice treated with vandetanib for 13 weeks. BIM expression was elevated in the lungs of both vehicle-treated and vandetanib-treated transgenic animals compared to non-transgenic animals. However, BIM expression was markedly lower in the 20-week-old vandetanib-treated transgenic mice, while Bcl-xL expression was similar in all treatment groups (Fig. 6). These data suggest that the suppression of BIM expression is one of the mechanisms associated with vandetanib resistance.

Discussion

Vandetanib, a tyrosine kinase inhibitor of VEGFR, EGFR, and RET signaling, suppressed lung adenocarcinoma in transgenic mice carrying the activating in-frame del *Egfr* E748-A752 mutation. Administration of the drug inhibited both EGFR signaling and neovascularization and significantly prolonged the survival of these animals. However, the lung adenocarcinomas of

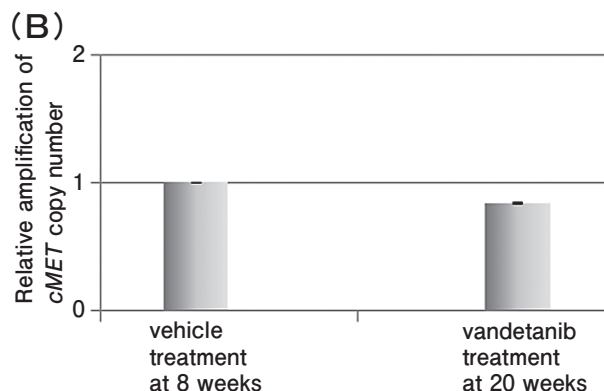
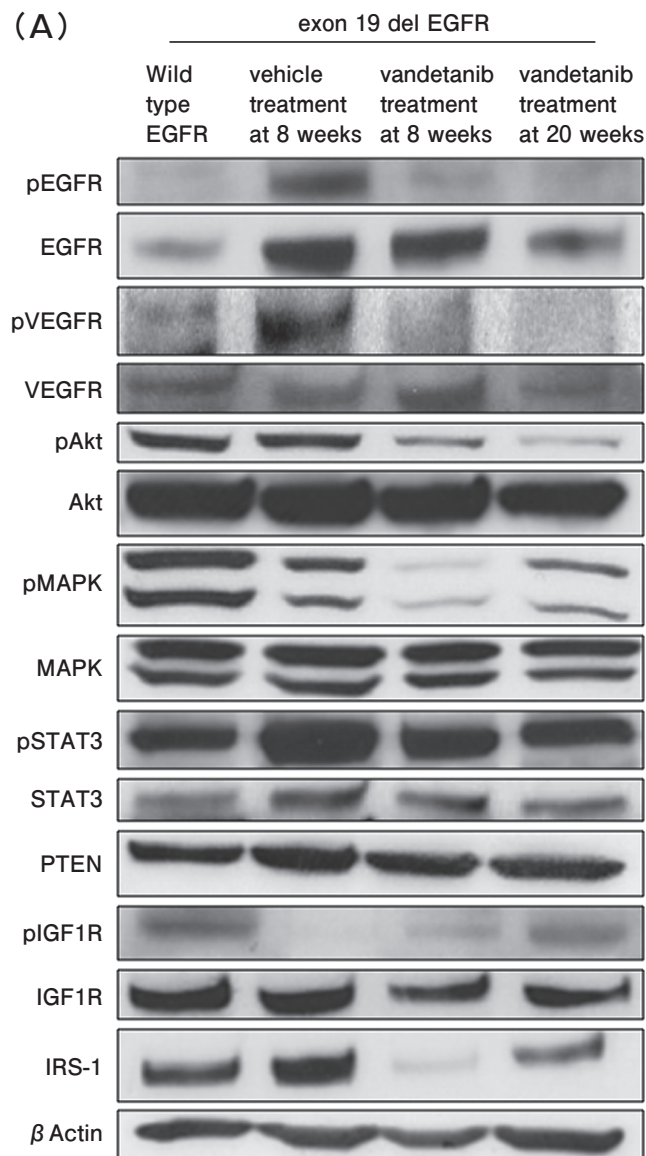


Fig. 5 **A**, Total EGFR, pEGFR, total VEGFR2, pVEGFR2, total Akt, pAkt, total MAP kinase, pMAP kinase, total STAT3, pSTAT3, PTEN, pIGF1R, and total IGF1R levels in the lungs of mice with wild-type *Egfr*, 8-week-old transgenic mice treated with vehicle or vandetanib for 1 week, and 20-week-old transgenic mice treated with vandetanib for 14 weeks; **B**, The *cMet* copy number in the lungs of the transgenic mice was determined. Genomic DNAs extracted from the lungs of 8-week-old mice treated with vehicle for 1 week and 20-week-old transgenic mice treated with vandetanib for 14 weeks were compared using TaqMan real-time PCR.

the transgenic mice eventually acquired resistance to vandetanib although none of the well-known mechanisms of resistance—for example, those involving EGFR-TKI, T790M, *cMet* amplification or PTEN loss—were detected.

Preclinical studies have demonstrated the efficacy of vandetanib in the treatment of lung adenocarcinoma, based on the results of orthotopic and subcutaneous xenograft experiments [32–34]. Tumors in the lungs of our transgenic mice occurred in a more physiologically relevant setting than is the case for xenograft tumors. In the transgenic mice, vandetanib inhibited

tumor growth by blocking EGFR and VEGFR-2 signaling and significantly prolonged survival. These data suggest that vandetanib has therapeutic potential for patients with lung cancer whose tumors harbor an activating *EGFR* mutation, and clinical trials with vandetanib are under way. Phase I/II clinical studies with vandetanib established the efficacy of a once-daily oral dose of up to 300 mg [17, 18]. The dose of vandetanib administered in the present study (6 mg/kg) was selected to achieve clinical relevance, since it yielded plasma levels of the drug that were within the range of those achieved in a group of Japanese patients

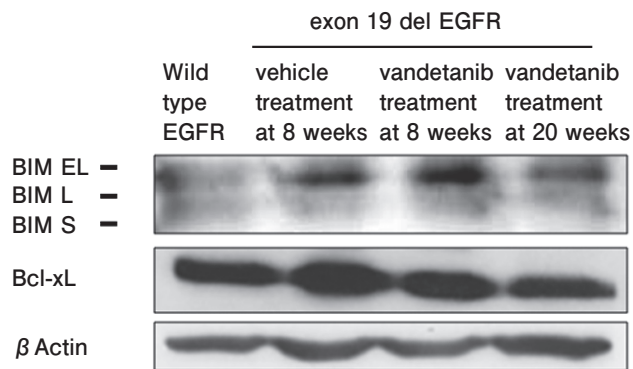


Fig. 6 BIM and Bcl-xL levels in the lungs of 8-week-old mice with wild-type *Egfr*, 8-week-old transgenic mice treated with vehicle, 8-week-old transgenic mice treated with vandetanib, and 20-week-old transgenic mice treated with vandetanib. The BIM gene encodes three major isoforms: BIM short (BIM_S), BIM long (BIM_L) and BIM extra long (BIM_{EL}).

with NSCLC administered 100–300 mg vandetanib/day [17, 35].

Among the known mechanisms of acquired resistance to EGFR-TKIs, one of the most important is a secondary mutation, T790M, in the EGFR ATP-binding site. In an *ex vivo* experiment [34], vandetanib was shown to select or induce the T790M mutation, but in our transgenic mouse model, the mutation could not be detected. Moreover, vandetanib reduced pEGFR in vandetanib-resistant tumor cells in the lungs. Thus, vandetanib resistance likely arises from events downstream of EGFR signaling or through the actions of another, unregulated growth factor receptor, not through low level competition from a vandetanib-ATP-binding site of EGFR other than T790M. MET amplification and PTEN inactivation were not detected, but pIGF-1R, IRS, and pMAPK were overexpressed. IGF-1R stimulates cell proliferation and survival by activating the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and Akt pathways [36–40]. IGF-1R activation may be associated with pMAPK activation and thus involved in vandetanib resistance. However, this remains to be evaluated in further experiments investigating the role of IGF-1R inhibition in transgenic mice with acquired resistance to vandetanib.

Recent studies have revealed that the pro-apoptotic BH3-only protein BIM mediated EGFR-TKI-induced apoptosis in lung cancers with oncogenic EGFR muta-

tions [28, 30, 31]. BIM is essential for tumor cell killing, and the shutdown of the EGFR-ERK signaling cascade is critical for BIM activation. In our mouse model, BIM was elevated in 8-week-old vandetanib-treated mice compared to mice of the same age treated with vehicle. BIM may mediate vandetanib-induced apoptosis in lung adenocarcinomas, a conclusion supported by the fact that the expression of this protein was suppressed in vandetanib-resistant mice. Therefore, one of the mechanisms of vandetanib resistance may be anti-apoptosis, in association with the ERK signaling cascade and suppression of BIM.

In summary, dual blockade of the EGFR and VEGFR signaling pathways by the small molecule inhibitor vandetanib has significant effects on lung adenocarcinomas induced by exon 19 del EGFR (deletion mutation E748–A752).

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