DDX3X mediates EGFR-TKI resistance through VEGFR signaling

Aya Ohtsubo
Niigata University Graduate School of Medical and Dental Sciences
Hiroshi Kagamu
Department of Respiratory Medicine, Saitama Medical University International Medical Center
Satoshi Watanabe (satoshi7@med.niigata-u.ac.jp)
Niigata University Graduate School of Medical and Dental Sciences
Kunihiro Shono
Niigata University Graduate School of Medical and Dental Sciences
Takaaki Masuda
Niigata University Graduate School of Medical and Dental Sciences
Tomoki Sekiya
Niigata University Graduate School of Medical and Dental Sciences
Ryo Suzuki
Niigata University Graduate School of Medical and Dental Sciences
Naohiro Yanagimura
Niigata University Graduate School of Medical and Dental Sciences
Satoshi Shoji
Niigata University Graduate School of Medical and Dental Sciences
Koichiro Nozaki
Niigata University Graduate School of Medical and Dental Sciences
Tomohiro Tanaka
Niigata University Graduate School of Medical and Dental Sciences
Yu Saida
Niigata University Graduate School of Medical and Dental Sciences
Rie Kondo
Niigata University Graduate School of Medical and Dental Sciences
Kenjiro Shima
Niigata University Graduate School of Medical and Dental Sciences
Yosuke Kimura
Niigata University Graduate School of Medical and Dental Sciences
Satoshi Hokari
Niigata University Graduate School of Medical and Dental Sciences
Nobumasa Aoki

Niigata University Graduate School of Medical and Dental Sciences
Yasuyoshi Ohshima
Niigata University Graduate School of Medical and Dental Sciences
Toshiyuki Koya
Niigata University Graduate School of Medical and Dental Sciences
Toshiaki Kikuchi
Niigata University Graduate School of Medical and Dental Sciences

Research Article

Keywords: VEGFR2, DDX3X, EGFR-TKI

Posted Date: December 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1073662/v1

Abstract

Although epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are remarkably effective against non-small-cell lung cancer (NSCLC) with *EGFR*-activating mutations, lung cancer cells acquire resistance to EGFR-TKIs without exception. Several mechanisms of EGFR-TKI resistance have been reported, but there are many aspects that remain to be clarified. We previously identified DDX3X as an immunogenic protein preferentially expressed in murine melanoma with a cancer stem cell (CSC)-like phenotype. DDX3X induced epithelial-mesenchymal transition and reduced the sensitivity to EGFR-TKIs in PC9 cells, human lung cancer cells harboring *EGFR* exon 19 deletion. We also reported that there was a small nonadherent subpopulation of parental PC9 cells that highly expressed DDX3X and had CSC properties. In this study, we found that VEGFR2 was upregulated in lung cancer cells that strongly expressed DDX3X and that these cells were addicted to VEGFR signaling. The blockade of both EGFR and VEGFR signaling reduced the phosphorylation of downstream signals in the cells with DDX3X that acquired EGFR-TKI resistance. The addition of VEGFR-TKIs or anti-VEGF antibodies to EGFR-TKIs significantly inhibited the progression of *EGFR*-mutated NSCLC in a xenograft mouse model. These data suggest that the blockade of VEGFR signaling enhances the antitumor effects of EGFR-TKIs by eradicating cancer stem cells, which mediate resistance to EGFR-TKIs.

Introduction

Mutations in the epidermal growth factor receptor (EGFR) have been shown to increase the kinase activity of EGFR and over-activate the downstream pro-survival signaling pathway [1,2]. In the presence of ligand-independent activation of EGFR signaling, cancer cells become dependent on EGFR signaling for pro-survival signaling pathways. Treatments targeting this signaling dependence have led to breakthrough therapeutic outcomes in the clinical setting. The use of EGFR tyrosine kinase inhibitors (TKIs) has significantly improved progression-free survival (PFS) in non-small-cell lung cancer (NSCLC) patients harboring activating *EGFR* mutations; however, lung cancer eventually acquires resistance and recurs without exception [3,4]. Most cases of acquired resistance reflect the selection of cancer cells harboring stochastic resistance-conferring genetic alterations. The mechanisms of EGFR-TKI resistance include *T790M* mutation, *c-Met* amplification, *PIK3CA* mutation and epithelial-to-mesenchymal transition (EMT) [5], which account for 50-70% of cases of resistance, but the remaining mechanisms remain unknown [6].

Recent evidence has demonstrated that cancer stem cells (CSCs) are critically involved in resistance to cytotoxic therapies. Sharma et al. reported that a small subpopulation of reversibly drug-tolerant cells existed in all examined cancer cells and that the drug-tolerant cells behaved as mother cells, giving rise to drug-resistant cells harboring additional mutations [7]. This small subpopulation shares biological characteristics with stem cells, is able to survive lethal stresses such as chemotherapy and radiotherapy and plays an important role in cancer treatment.

DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked (DDX3X) is a member of the DEAD-box family of ATP-dependent RNA helicases and is located on the X chromosome [8]. DEAD-box helicases have

multiple functions in processes including RNA splicing, mRNA export, transcriptional and translational regulation, RNA decay, ribosome biogenesis, and miRNA regulation [9,10]. Thus, DDX3X is thought to be involved in the epigenetic regulation of gene expression. Our previous proteome analyses identified DDX3X as a protein preferentially expressed in purified $CD133^+$ B16 melanoma cells, which possess CSC-like properties [11,12]. Recently, we demonstrated that *EGFR*-mutated NSCLC cells with *DDX3X* transgenes lost EGFR signaling addiction and acquired EGFR-TKI resistance without any additional *EGFR* mutations. Moreover, we found that a small subpopulation of parental lung cancer cells with a mesenchymal cell phenotype strongly expressed DDX3X and exhibited EGFR-TKI resistance [13].

In this study, we sought to investigate alternative prosurvival signaling pathways induced by DDX3X in EGFR-TKI-resistant lung cancer cells and found that vascular endothelial growth factor receptor 2 (VEGFR2) expression was increased in DDX3X-expressing lung cancer cells. Recent evidence has shown that anti-VEGF therapy significantly enhances the antitumor efficacy of EGFR-TKIs in *EGFR*-mutated NSCLC patients [14,15]. In addition, VEGF-mediated signaling has been shown to contribute to key aspects of tumorigenesis, including the self-renewal and survival of CSCs [16]. Therefore, we investigated whether signaling through VEGFR is involved in EGFR-TKI resistance in lung cancer cells expressing DDX3X.

Results

High expression of DDX3X reduces EGFR signaling in cancer cells harboring EGFR**-activating mutations.**

To examine the effects of DDX3X on the cellular phenotype, we transfected PC9 cells, human lung adenocarcinoma cells harboring an *EGFR* exon 19 deletion mutation, with cDNA encoding *DDX3X* and established a novel cell line, termed A-4, which overexpressed DDX3X. Immunoblotting analysis confirmed the overexpression of DDX3X in the transfectants (Fig. 1A).

The survival and proliferation of PC9 cells are highly dependent on EGFR signaling because PC9 cells possess driver mutations in the *EGFR* gene that enhance tyrosine kinase activity. We analyzed whether EGFR signaling was affected by the overexpression of DDX3X. As shown in Fig. 1B, EGFR phosphorylation was observed in parental PC9 cells but not in A-4 cells. The suppression of EGFR phosphorylation was also observed in A-1, A-2 and A-5 cells transfected with DDX3X (Fig. 1B).

Our previous study demonstrated that the CSC-like subpopulation of PC9 cells expressed DDX3X and exhibited scaffold-independent growth [13]. To examine whether the overexpression of DDX3X affects the scaffold-independent growth of lung cancer cells, the ratio of nonadherent to adherent cells among parental PC9, A-1 and B-2 cells (mock) was evaluated. The ratio of nonadherent cells to adherent cells significantly increased in A-1 cells, suggesting that DDX3X promotes adhesion-independent proliferation (Fig. 1C). Because nonadherent cells were also present among parental PC9 cells, we assessed the expression of DDX3X in parental nonadherent PC9 cells. Similar to A-4 cells, nonadherent PC9 cells showed DDX3X overexpression (Fig. 1D). In addition, nonadherent PC9 cells lacked EGFR phosphorylation despite the presence of EGF (Fig. 1E). The subsequent phosphorylation of extracellular

signal-regulated kinase (ERK) and serine/threonine kinase (AKT) in nonadherent PC9 and A-4 cells was also suppressed.

Nonadherent cells overexpressing DDX3X express VEGFR2.

Recent clinical trials showed that the addition of anti-VEGF therapy to EGFR-TKIs prolonged PFS in NSCLC patients with activating *EGFR* mutations [14,15]. Therefore, we hypothesized that VEGF might be involved in EGFR-TKI resistance and performed FACS analysis to determine whether VEGFR was expressed on the surface of *EGFR*-mutated NSCLC cells. The results showed that approximately 50% of the nonadherent population of parental PC9 cells, which overexpressed DDX3X, strongly expressed VEGFR2, while only approximately 1% of adherent cells among PC9 parental cells expressed VEGFR2 (Fig. 2).

The combination of EGFR-TKIs and VEGFR-TKIs inhibits the phosphorylation of ERK and AKT in DDX3X-overexpressing cells.

We next performed immunoblotting analysis to investigate the influence of VEGFR-TKIs on EGFR downstream signaling. In the parental PC9 cells, the EGFR-TKI erlotinib alone inhibited the phosphorylation of ERK and AKT, whereas the VEGFR-TKI lenvatinib alone did not interfere with the phosphorylation of ERK and AKT in PC9 cells (Fig. 3A and B). In A-4 cells, neither erlotinib nor lenvatinib alone reduced the phosphorylation of ERK and AKT (Fig. 3A and B). In contrast, the combination of erlotinib and lenvatinib reduced the phosphorylation of ERK and AKT in A-4 cells (Fig. 3A and B).

The combination of EGFR-TKIs and VEGFR-TKIs or anti-VEGF antibodies had synergistic effects in DDX3X high-expressing cells in vivo.

To investigate the antitumor effect of the combination of EGFR-TKIs and VEGFR-TKIs in vivo, we generated a mouse xenograft tumor model with PC9 and A-4 cells. As shown in Fig. 4A, the progression of A-4 skin tumors was faster than that of PC9 skin tumors. Therefore, each drug treatment was started on day 21 for the PC9 cell groups and on day 14 for the A-4 cell groups, and treatments for both groups were continued for 2 weeks. In the PC9 cell group, tumor growth was significantly inhibited by erlotinib (Fig. 4B). On the other hand, in the A-4 cell group, erlotinib appeared to inhibit tumor growth slightly in the early phase of drug administration, but tumor growth was generally the same as that in the untreated group (Fig. 4C). However, the addition of lenvatinib to erlotinib markedly delayed the growth of tumors derived from A-4 cells (Fig. 4D). We next investigated whether bevacizumab, an anti-VEGF monoclonal antibody (mAb), could exert a synergistic antitumor effect when used in combination with erlotinib. As shown in Fig. 4E, the combination of bevacizumab and erlotinib showed significantly greater antitumor effects than erlotinib alone in A-4 cells. The combination of lenvatinib and erlotinib also significantly inhibited the progression of tumors derived from PC9 cells compared to that with erlotinib alone (Fig. 4F).

Discussion

Our previous study demonstrated that strong expression of DDX3X in lung cancer cells with *EGFR*-activating mutations was accompanied by CSC-like properties, EMT and resistance to EGFR-TKIs due to the loss of EGFR signaling addiction [13]. In the current study, we investigated alternative prosurvival signals induced by DDX3X in the context of EGFR-TKI resistance using human lung cancer cell lines. We found that DDX3X induced the upregulation of VEGFR2 and signal transduction by VEGFR2. An in vivo xenograft model demonstrated that dual inhibition of EGFR and VEGFR signaling had synergistic durable therapeutic effects on DDX3X-expressing cancer cells.

CSCs can survive under potentially lethal stress conditions because they have multiple mechanisms by which they can resist cell death, including altered chromatin status; overexpression of multidrug efflux transporters, antiapoptotic factors, or DNA repair gene products; and stem cell-specific growth signaling [7,17–22]. Recent studies have demonstrated that CSCs are involved in TKI resistance [23,24] and that the suppression of CSC properties could inhibit EGFR-TKI resistance in lung adenocarcinoma [25]. The present study showed that forced expression of DDX3X in lung cancer cells resulted in the suppression of EGFR phosphorylation, suggesting that these DDX3X-expressing cells proliferate independently of EGFR signaling (Fig. 1B). Indeed, lung cancer cells with high expression of DDX3X showed resistance to erlotinib in vivo (Fig. 4C). These DDX3X-expressing A-4 cells showed anchorage-independent proliferation (Fig. 1C). Parental PC-9 cells also included nonadherent cells, which showed a high level of DDX3X expression and a reduction in EGFR signaling (Fig. 1D and E). These findings indicated that *EGFR*-mutated lung cancer cells include CSC-like cells that strongly express DDX3X and that these cells play an important role in resistance to EGFR-TKIs.

Recent basic and clinical studies have demonstrated the importance of dual inhibition of EGFR and VEGFR in *EGFR*-mutated lung cancer cells. Cancer cells express VEGFRs, and autocrine VEGF/VEGFR signaling promotes cancer cell growth, survival, migration, and invasion [26]. VEGF expression is driven by many factors that are characteristic of tumors, including the expression of oncogenes, (e.g., ras, src, ERBB2, and EGFR) and hypoxia, and the EGFR and VEGF/VEGFR signaling pathways are tightly connected with each other [27]. Preclinical studies have shown that dual inhibition of EGFR and VEGFR-dependent signaling overcomes the resistance to EGFR-TKIs [28,29]. Randomized phase III studies have demonstrated that combination therapy with bevacizumab or ramucirumab and erlotinib significantly prolongs PFS compared with erlotinib monotherapy [14,15]. VEGF has been found to be involved in the promotion of cancer stemness and self-renewal; VEGF/VEGFR2/neuroplilin-1 autocrine signaling regulates CSCs to promote glioma growth [30], and in skin cancer, the blockade of VEGFR2 not only decreased the microvascular density but also reduced pool size and impaired CSC renewal properties, resulting in tumor regression [31]. Additionally, in 2015, Zhao et al. showed that VEGF promotes CSC self-renewal in breast and lung cancers through VEGFR2/STAT3-mediated upregulation of Myc and Sox2 [32]. In the current study, combination therapy with VEGFR-TKI or anti-VEGF antibodies and EGFR-TKI significantly delayed the progression of lung cancer cells with CSC-like properties (Fig. 4D and E). Previous studies showed that the mechanisms of anti-VEGF therapy were normalization of intratumor blood flow and improvement of drug delivery [33,34]. We demonstrated that in lung cancer cells that

overexpressed DDX3X and acquired CSC-like characteristics, EGFR-TKIs or VEGFR-TKIs alone did not suppress downstream signaling; however, the combined use of VEGFR-TKIs with EGFR-TKIs suppressed the phosphorylation of ERK and AKT (Fig. 3A and B). Because these results were observed in in vitro experiments without stromal cells, the blockade of signaling through VEGFR2 in EGFR-TKI-resistant cells is one of the mechanisms underlying the effectiveness of anti-VEGF therapy.

In conclusion, this study demonstrates that a DDX3X-dependent increase in VEGFR2 expression may confer EGFR-TKI resistance in lung cancer cells with EGFR-activating mutations. Combination therapy with EGFR-TKIs and VEGFR-TKIs seems to be a promising approach to overcome EGFR-TKI resistance mechanisms based on the eradication of CSCs.

Materials And Methods

Tumors

PC9 human lung adenocarcinoma cells harboring an *EGFR* exon 19 deletion were obtained from Riken BioResource Center and maintained in culture medium containing RPMI 1640 medium supplemented with 10% heat-inactivated lipopolysaccharide-qualified fetal calf serum, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate (all from Life Technologies, Inc., Tokyo, Japan) [13].

Transfection of PC9 cells with *DDX3X* cDNA

Transfection of PC9 cells with *DDX3X* cDNA was obtained using a Myc-DDK-tagged open reading frame clone of *Homo sapiens* DDX3X transcript variant 1 as transfection-ready DNA (Origene Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocol [13]. Experimental cells were incubated with fresh medium containing G418 (600 μg/mL, Promega, Madison, WI, USA), and the medium was replaced with fresh G418-containing medium every 3–4 days until resistant colonies were identified [13].

Immunoblotting

Cells were harvested and lysed in Nonidet P-40 buffer containing a protease-inhibitor mixture (Sigma, St. Louis, MO, USA) [12]. Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA, USA) and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) [13]. Immunoblots of tumor cell lysates were probed with antibodies against DDX3X (Sigma-Aldrich, St. Louis, MO, USA), EGFR, phospho-EGFR (Tyr1068), phospho-EGFR (Tyr1173), phospho-EGFR (Tyr845), Akt, phospho-Akt, Erk1/2, phospho-Erk1/2, and β-actin (Abnova, Taipei, Taiwan) [12,13]. All antibodies except for anti-DDX3X and anti-β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA) [13]. The secondary antibody was either anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Immunoreactive protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA) [12].

Cell viability assessment

Cells were cultured on 24-well plates for 3 days [13]. Nonadherent cells were collected by gentle rocking in culture medium [13]. The ratio of nonadherent cells to adherent cells was calculated [13]. Dead cells were excluded via the trypan blue exclusion method [13].

Monoclonal antibodies and flow cytometry

PE-conjugated anti-VEGFR2 (Avas 12α1) antibody was purchased from BD Biosciences (San Jose, CA, USA). Analysis of cell-surface phenotypes was carried out by direct immunofluorescent staining of 0.5–1×10^6 cells with conjugated monoclonal antibodies and then treatment with 1% paraformaldehyde [12]. In each sample, 10,000 cells were analyzed using a FACSCalibur flow microfluorometer (BD Biosciences) [12]. PE-conjugated subclass-matched antibodies used as isotype controls were also purchased from BD Biosciences [12]. The samples were analyzed with CellQuest software (BD Biosciences) [12].

Animals

Female C.B-17/Icr-scid/scidJcl (SCID) mice were purchased from CLEA (Japan), maintained in a specific-pathogen-free environment, and used for experiments at the age of 10 weeks. The experimental protocols were based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Niigata University Institutional Animal Care and Use Committee. At the end of the experiment, the mice were euthanized due to cervical dislocation. This study was reported in accordance with the ARRIVE guidelines.

Adoptive medication therapy

SCID mice were injected s.c. with parental PC9 or DDX3X-transfected PC9 (PC9-DDX3X) tumor cells in 100 μl of Hanks' balanced salt solution to establish skin tumors. Two or three weeks after inoculation, each group was randomized to groups of five mice each, and vehicle, 25 mg/kg erlotinib (Selleck Chemicals, Houston, Texas, USA), 25 mg/kg lenvatinib (Selleck Chemicals, Houston, Texas, USA) or both was given intraperitoneally to the mice every 5 days a week. Bevacizumab was administered at a dose of 5 mg/kg three times a week. The perpendicular diameter of skin tumors was measured with digital calipers.

Statistical analysis

For experiments in the animal tumor model, the significance of differences between groups was analyzed using Student's t-test. All other experimental data were analyzed by one-way ANOVA, and each group was compared by Bonferroni's multiple comparison test. All statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [35]. A 2-tailed P value of < 0.05 indicated statistical significance. All experiments were repeated at least twice.

Declarations

Data availability

The data generated or analyzed during the current study are available from the corresponding authors on reasonable request.

Acknowledgments

The authors want to thank Hiroko Aita for their professional assistance.

Author contributions

A.O., S.S. and K.N. performed the experiments. H.K. conceived the study. S.W. managed the study. T. Koya was involved in the analysis. T.S., K. Shono, T.M., R.S., T.T., Y.S., R.K., K. Shima, Y.K., S.H., N.A. and Y.O. revised the paper. T. Kikuchi supervised the work. All authors read and approved the final manuscript.

Conflict of interest statement: The authors declare no competing interests.

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Figures

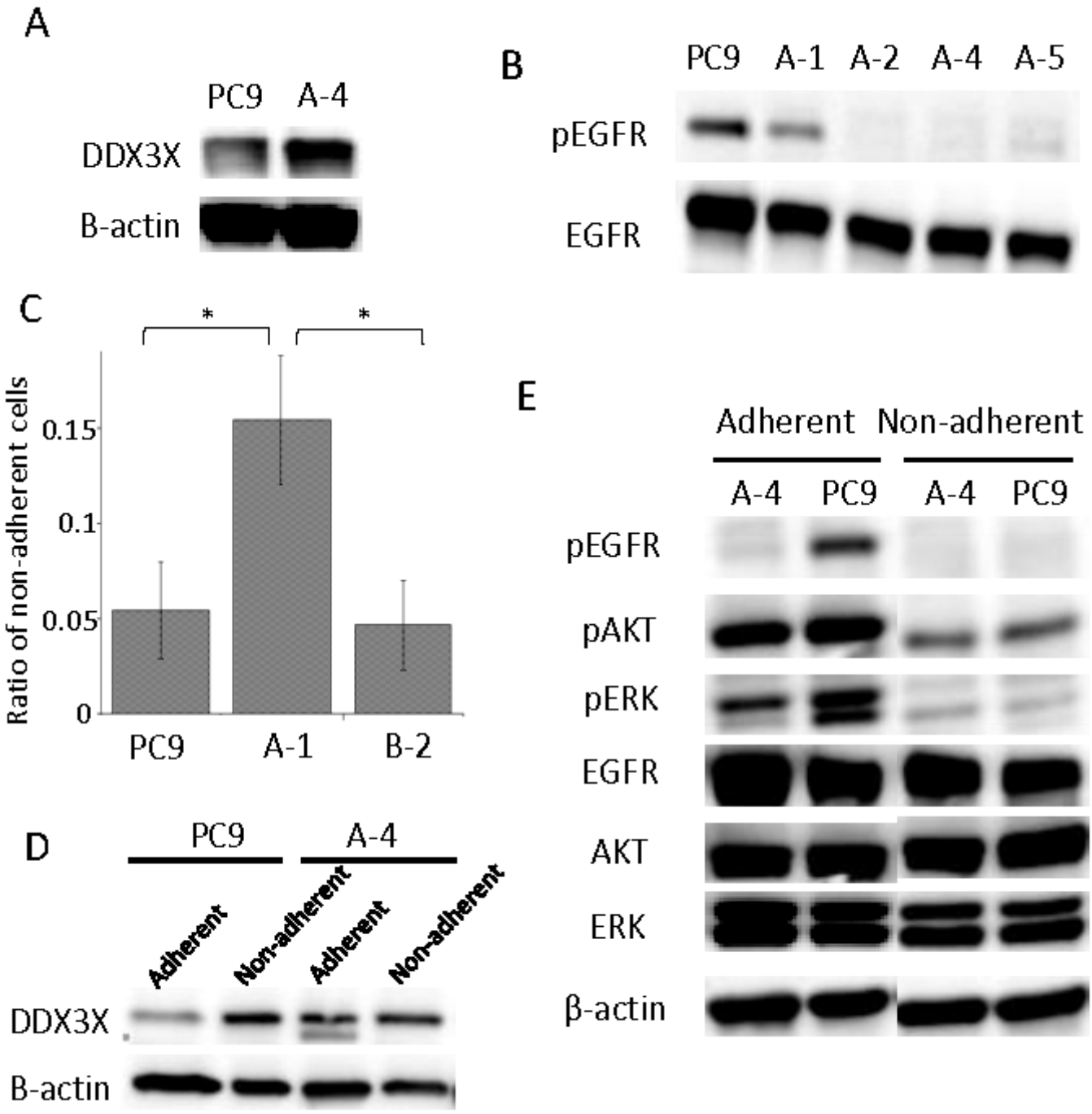

Figure 1

DDX3X reduced EGFR signaling. We generated PC9 cells that overexpressed DDX3X and named them A-1, A-2, A-4, and A-5 cells. (A) Immunoblotting analysis of DDX3X in parental PC9 cells and A-4 cells. (B) Immunoblotting analysis of phospho-EGFR and EGFR in parental PC9 cells and A-1, A-2, A-4 and A-5 cells. (C) Viable cells counts of 1×105 PC9 cells, A-1 cells and B-2 cells (mock) cultured for 3 days in 24-well plates. *P<0.05. Data are presented as the mean ± SD of three independent experiments. (D) Immunoblotting analysis of DDX3X in adherent and nonadherent parental PC9 cells and adherent and nonadherent A-4 cells. (E) Immunoblotting analysis of phospho-EGFR, phospho-AKT, phospho-ERK, EGFR,

AKT and ERK in the presence of EGF in adherent and nonadherent parental PC9 cells and adherent and nonadherent A-4 cells.

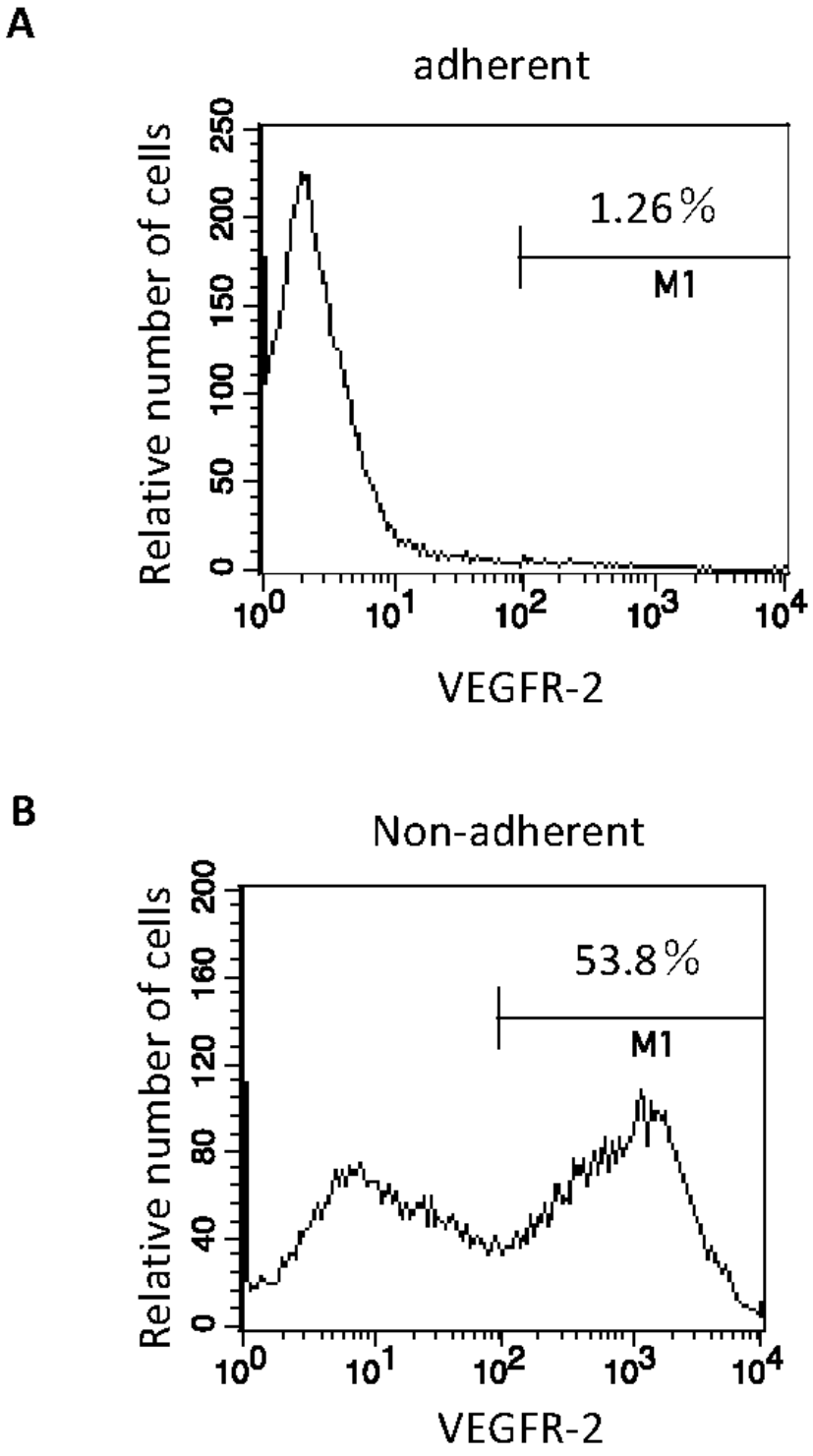

Figure 2

VEGFR2 expression rate. Flow cytometry analysis of VEGFR2 expression in adherent and nonadherent parental PC9 cells.

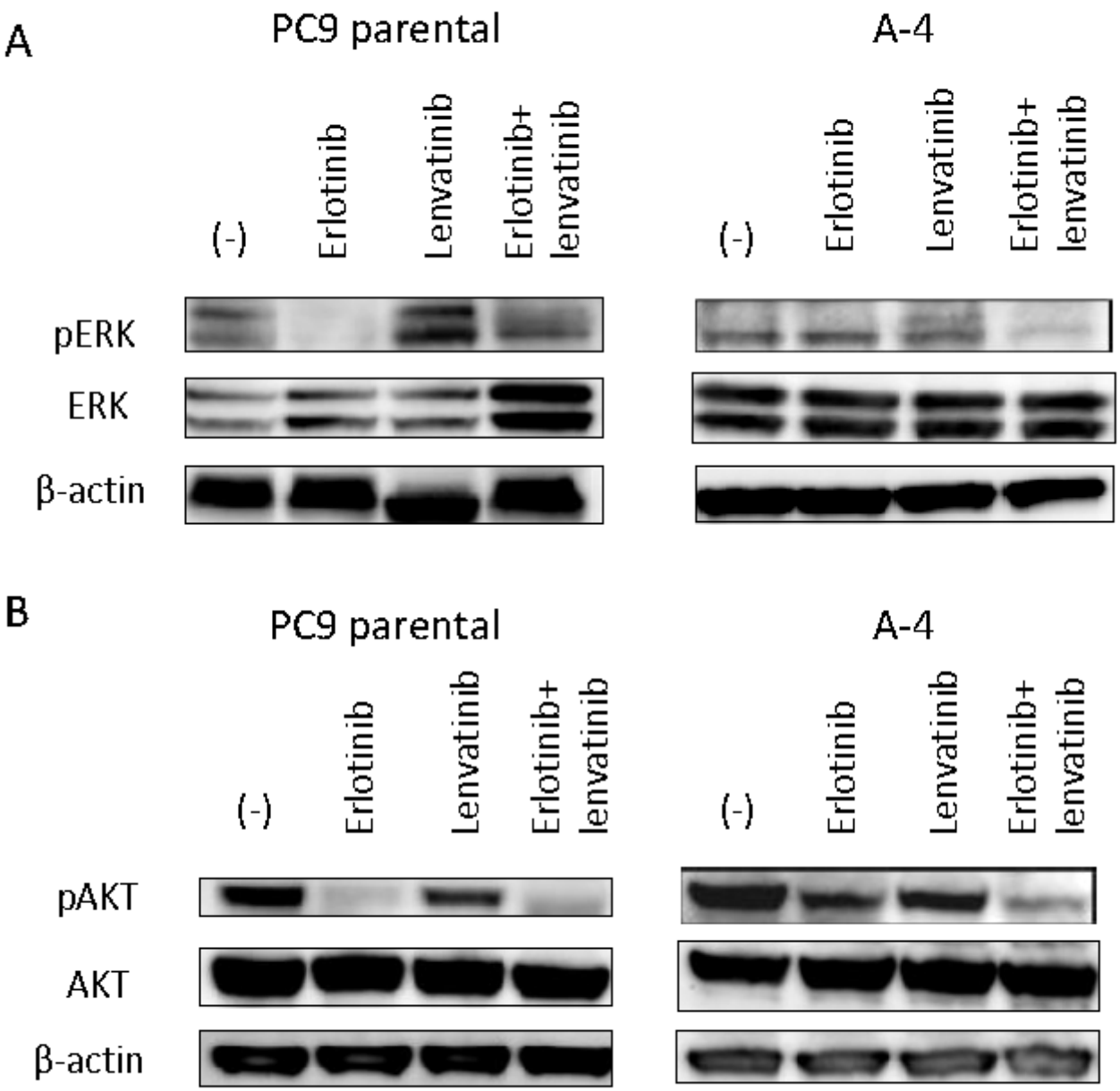

Figure 3

Inhibition of ERK and AKT phosphorylation by the combination of EGFR-TKIs and VEGFR-TKIs. (A-B) Immunoblotting analysis of phospho-ERK, ERK, phospho-AKT and AKT in parental PC9 cells and A-4 cells. Each cancer cell line was treated with the indicated concentrations of erlotinib and lenvatinib for 24 hours.

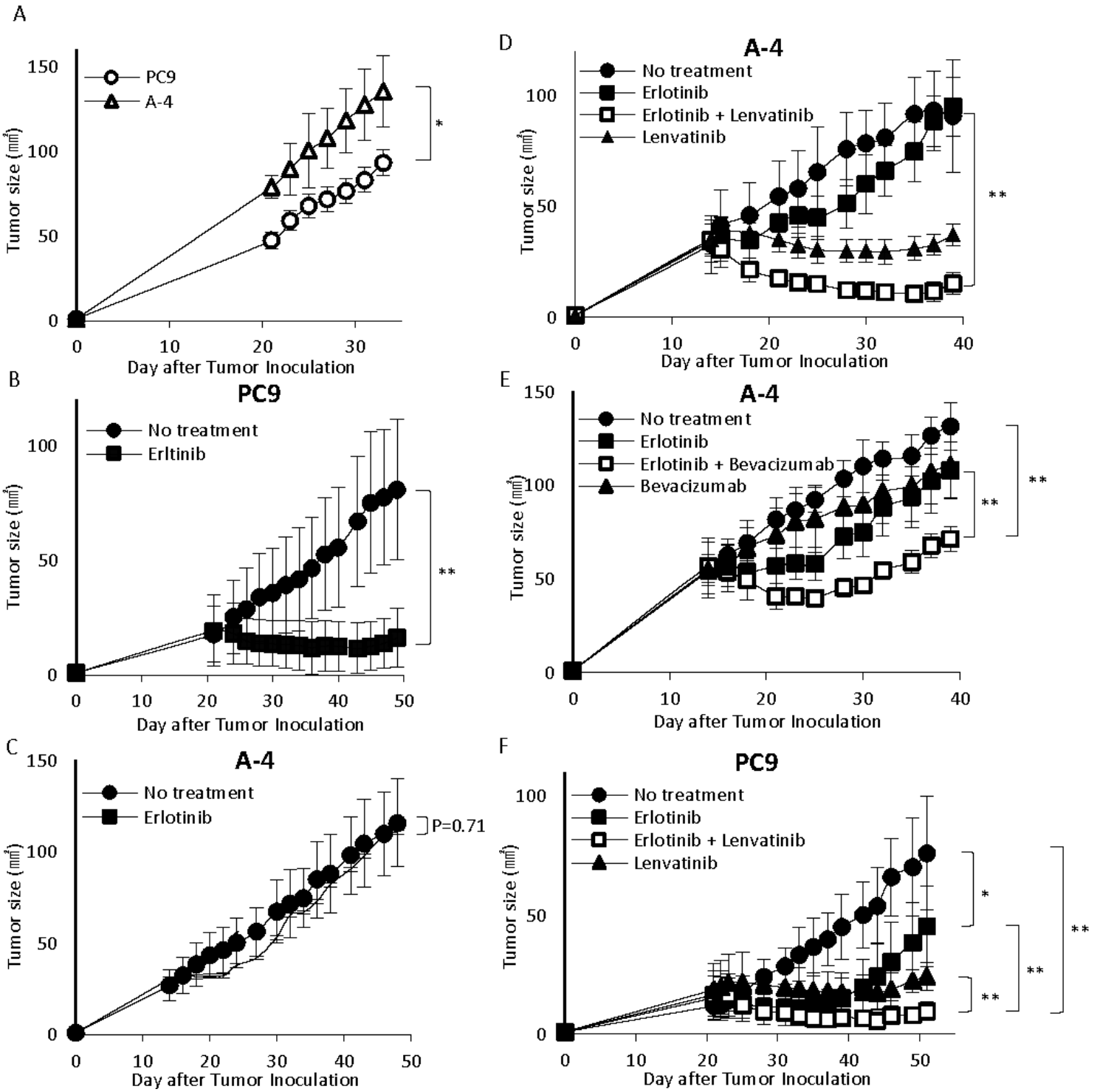

Figure 4

Antitumor effects of EGFR-TKIs combined with VEGFR-TKIs or anti-VEGF antibody therapy in vivo. SCID mice were inoculated s.c. with PC9 or A-4 cells, and the tumor areas (mm2) were recorded. (A) Observation of tumor growth in the PC9 and A-4 groups. *P<0.05. Data are presented as the mean ± SD of three independent experiments. (B-C) Mice were assigned to two groups of five mice each and treated with vehicle or 25 mg/kg erlotinib i.p. 5 days a week for 2 weeks, 3 weeks after inoculation of PC9 cells and 2 weeks after inoculation of A-4 cells. **P<0.01. Data are presented as the mean ± SD of three

independent experiments. (D-F) Mice were randomly divided into 4 groups of 5 mice each and treated with vehicle, 25 mg/kg erlotinib, 25 mg/kg lenvatinib or both i.p. 5 days a week. Bevacizumab was administered at a dose of 5 mg/kg 3 times a week, 2 weeks after the inoculation of A-4 cells and 3 weeks after inoculation of PC9 parent cells. *P<0.05. **P<0.01. Data are presented as the mean ± SD of three independent experiments.