

Anlotinib Exerts Anti-Cancer Effects on KRAS-Mutated Lung Cancer Cell Through Suppressing the MEK/ERK Pathway

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Background: With a high frequency of 30%, KRAS mutations in patients with non-small cell lung cancer (NSCLC) often lead to their poor response to most anti-cancer therapies. As a multi-target tyrosine kinase inhibitor, Anlotinib shows clinical efficacy against several types of cancer. However, its effects on KRAS mutant NSCLC and the underlying molecular mechanisms remain unclear.

Materials and Methods: Cell counting Kit-8 assay, colony formation assay, flow cytometry analysis, wound healing scratch assay, Transwell assay and xenograft mouse model were used to evaluate the anti-cancer effects of Anlotinib. The potential molecular mechanisms were determined by immunohistochemistry (IHC) and Western blotting.

Results: Anlotinib inhibited proliferation of KRAS mutant lung cancer cells and induced apoptosis in vitro. In addition, the migration and invasion abilities of these cells were also decreased after treatment with Anlotinib. It significantly suppressed tumor growth in vivo and prolonged the survival of the xenograft-bearing mice, which correlated to lower expression levels of Ki67 in the tumor tissues. Mechanistically, Anlotinib downregulated MEK and ERK as well as their phosphorylated forms in the KRAS mutant lung cancer cells.

Conclusion: Anlotinib inhibits the growth of KRAS mutant lung cancer cells partly via the suppression of the MEK/ERK pathway. Our findings provide novel insights into treating recalcitrant KRAS mutated NSCLC.

Keywords: Anlotinib, KRAS-mutated, lung cancer, signal pathways

Introduction

Lung cancer is a commonly diagnosed malignancy associated with high morbidity and mortality. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases.¹ Cisplatin-based chemotherapy has been the mainstay of advanced NSCLC treatment, although the 5-year survival rate is less than 5%.² With the advent of targeted therapy, the prognosis and survival of NSCLC patients harboring mutations such as EGFR, ALK or ROS-1 have improved significantly.³

KRAS is commonly mutated in lung cancer and especially prevalent in NSCLC.^{4,5} Mutations in KRAS constitutively activate its downstream signaling pathways⁶ such as MEK/ERK, a mitogen-activated protein kinase (MAPK) pathway, this is known to promote cancer cell survival and chemoresistance.^{7,8} KRAS mutations correlate significantly to poor therapeutic response in NSCLC patients.^{9–11} Therefore, it is essential to develop novel KRAS-targeting drugs for treating recalcitrant NSCLC patients with KRAS mutations.

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Anlotinib is a novel tyrosine kinase inhibitor (TKI)¹² that targets multiple TKs including VEGFR, PDGFR, FGFR and c-Kit. It has shown potent therapeutic effects against renal cell carcinoma, thyroid cancer and osteosarcoma in preclinical and clinical trials. Wang showed that Anlotinib blocked osteosarcoma development and progression by inhibiting VEGFR2 and MET.¹³ Likewise, Lin and Xie reported anti-angiogenic effects of Anlotinib via suppression of VEGFR, PDGFR, FGFR and c-Kit.^{14,15} At present, Anlotinib is approved in China as a single drug therapy for patients with locally advanced or metastatic NSCLC who present tumor progression or recurrence after undergoing at least 2 lines of systemic chemotherapy.¹⁶ The Phase II (ALTER0302) and Phase III (ALTER0303) clinical trials conducted by Han showed that Anlotinib significantly improved the OS and PFS of patients with advanced NSCLC as a third-line treatment compared to the placebo, and was well tolerated.^{17–19} Si also reported that Anlotinib improved the quality of life in advanced NSCLC patients, and recommended it for the third-line and subsequent treatment.²⁰ Recently, a case report demonstrated that an advanced lung adenocarcinoma patient with KRAS mutation responded well to Anlotinib, thus indicating its suitability for recalcitrant KRAS mutant NSCLC. However, no preclinical studies so far have analyzed the effects of Anlotinib on KRAS mutant NSCLC or explored the possible molecular mechanisms. To this end, we evaluated the effects of Anlotinib on lung cancer cells harboring the KRAS mutation both in vitro and in vivo, and investigated the underlying molecular mechanisms.

Materials and Methods

Cell Culture

The human NSCLC cell lines A549 and NCI-H460 respectively harboring the G12S and Q61H KRAS mutations were purchased from American Type Culture Collection (ATCC). The cells were cultured at 37°C in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (Penicillin 100U/mL, Streptomycin 100 ug/mL) under 5% CO₂. All experiments were conducted in the exponential growth phase.

CCK8 Assay

The A549 and NCI-H460 cells were seeded in 96-well plates at the density of 3×10^3 cells/well and incubated overnight. Following treatment with different concentrations

(10, 20, 30, and 40 μM) of Anlotinib (China Tai Tianqing Pharmaceutical Group Co. Ltd.) for 48h, the CCK-8 reagent (Dojindo, Kumamoto, Japan) was added, and the cells were incubated for another hour at 37°C. The percentage of viable cells was determined by measuring the absorbance at 450 nm using a microplate reader (Bio-Rad, USA). The median inhibitory concentration (IC₅₀ value) was calculated using Prism 7.0 software (GraphPad Software).

Colony Formation Assay

The cells were seeded in 6-well plates at the density of 500 cells/well and incubated overnight. After culturing the cells with different concentrations of Anlotinib for 14 days, the ensuing colonies were stained with 0.1% crystal violet for 20 min. The number of colonies (> 50 cells) were counted under a Leica DM1400B inverted microscope linked to a camera.

Flow Cytometry

The cells were seeded in 6-well culture plates at the density of 2×10^5 cells/mL and after an overnight culture, treated with different concentrations of Anlotinib for 48h. After washing twice with PBS, the cells were harvested and stained with Annexin V + 7-AAD reagent (Keygen, Biotech, Nanjing, China) at room temperature for 15 min in the dark according to the manufacturer's protocol. The percentage of apoptotic cells was evaluated by flow cytometry (BD FACSCalibur; BD Bioscience).

Wound Healing Scratch Assay

The cells were seeded in 6-well plates and grown until confluent. The monolayer was scratched with a sterile pipette tip, and the dislodged cells were washed. Images of the "wound" region were taken at 0h and 48h of culture with different concentrations of Anlotinib under a camera-equipped light microscope (Leica, Japan).

Transwell Assay

Transwell chambers (8 μm pore size; Corning Costar, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA) were placed in a 24-well culture plate. The lower chambers were filled with 600 μL complete RPMI 1640 medium, and the cells were seeded in the upper chambers in 200 μL serum-free medium. After culturing the cells with different concentrations of Anlotinib for 48h, the transwell chambers were transferred into a fresh plate, washed with PBS, fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet (CV) for

15 min. The number of invasive cells were counted under a Leica DM1400B inverted microscope linked to a camera.

Western Blotting

The suitably treated A546 and NCI-H460 cells were homogenized with RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail (Beyotime Biotechnology). The protein concentration of the lysates was measured using bicinchoninic acid assay (BCA). Equal amounts of protein per sample were separated by 10% SDS-PAGE and then electro-transferred to a PVDF membrane for 40 min at 4°C. After blocking with 5% skimmed milk/Tris-Tween buffer saline (TBST) for 1h at

room temperature, the membranes were incubated overnight with the primary antibodies against ERK, p-ERK, MEK, p-MEK (1:1000, Cell Signaling Technology, CST), BAX, BCL-2 and β -actin (1:1000, Abcam, MA, USA). The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 60 min. The positive bands were detected using an electrochemiluminescence (ECL) reagent.

Xenograft Mouse Model

The animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of West China Hospital of Sichuan University, and approved by the Animal Ethics Committee of Sichuan University.

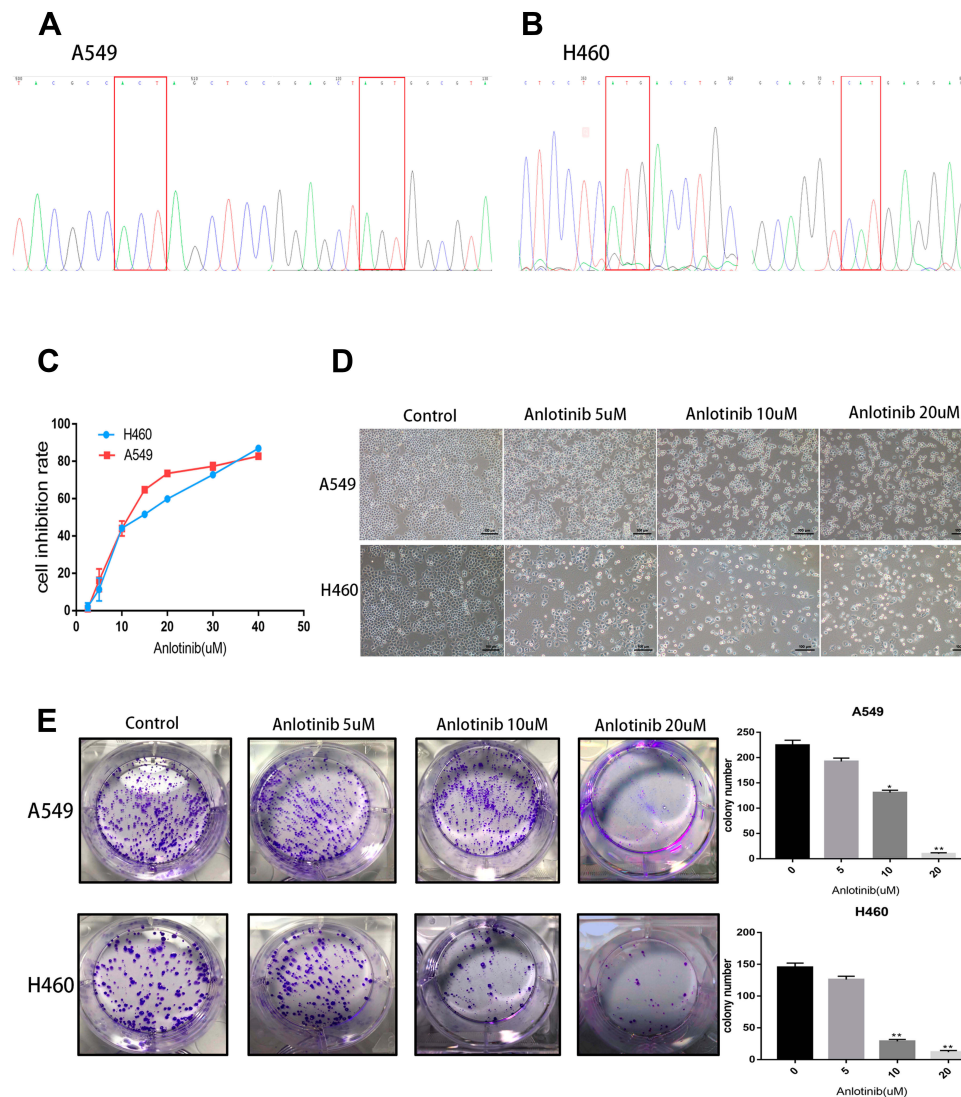


Figure 1 Anlotinib inhibits proliferation of KRAS mutant lung cancer cells. (**A** and **B**) KRAS mutation sites of A549 and NCI-H460 cells (G12S and Q61H, respectively). (**C**) Cell inhibition rate with Anlotinib treatment. (**D**) Phase contrast microphotograph images of lung cancer cells with or without Anlotinib for 48h. (**E**) Colony formation after Anlotinib treatment. * $P < 0.05$, ** $P < 0.01$

Forty female nude mice (five-weeks-old) were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). To establish the xenograft model, approximately 2×10^6 A549 or NCI-H460 cells were subcutaneously injected into the right flank of each mouse. Once the tumors grew to 50 mm^3 – 100 mm^3 , the mice were randomly divided into the A549 Ctrl, A549 Anlotinib, H460 Ctrl and H460 Anlotinib groups ($n=10$ each), and injected intraperitoneally with normal saline or 5 mg/kg Anlotinib once daily for 14 days. The tumors were measured on alternate days using calipers and the volume (mm^3) was calculated as

$\text{length}/2 \times \text{width}^2$. Five randomly selected mice from each group were sacrificed by cervical dislocation, and the tumors were harvested, weighed and processed for immunohistochemical analysis. The remaining were observed for 70 days, and a death was recorded when a mouse died during treatment or if the tumor volume reached 1500 mm^3 .

Immunohistochemistry (IHC)

The freshly resected tumors were immersed in formalin and fixed overnight, embedded in paraffin, and cut into $4\mu\text{m}$ -thick serial sections. After dewaxing and dehydration,

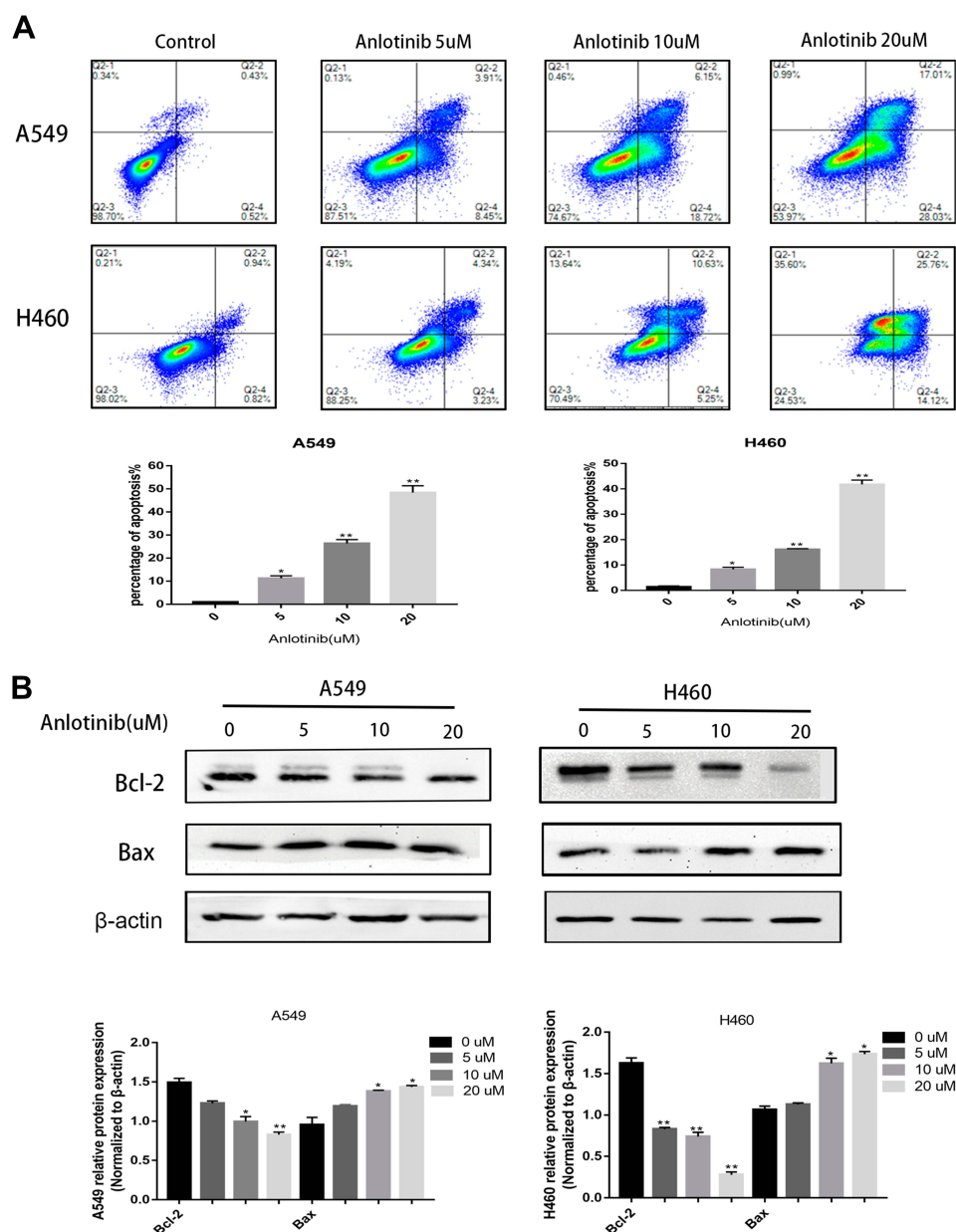


Figure 2 Anlotinib shows apoptosis induction in KRAS mutant lung cancer cells. **(A)** Flow cytometry detection of apoptotic cells after Anlotinib treatment. **(B)** Western blot analysis of Bcl-2 and Bax level after Anlotinib treatment. * $P<0.05$ ** $P<0.01$.

the sections were heated in citrate buffer for antigen retrieval. The processed sections were incubated with anti-CD31 and anti-Ki67 primary antibodies (1:1000, Abcam, MA, USA), followed by the secondary antibodies according to the manufacturer's instructions, and sealed with neutral gum. Vessel density was determined in terms of CD31 expression, and the percentage of Ki-67 positive proliferating cells was evaluated.

Statistical Analysis

Data are expressed as mean \pm standard deviation. Statistical analyses were performed using the Statistical Package for the

Social Sciences 24.0 (SPSS Inc., Chicago, IL, USA). The groups were compared using Dunnett's *t*-test, Student's *t*-test, or one-way analysis of variance with Student–Newman–Keuls ad hoc test as appropriate. $P < 0.05$ was considered statistically significant.

Results

Anlotinib Inhibits Proliferation of KRAS Mutant Lung Cancer Cells

The specific KRAS mutations in A549 and H460 cells were verified by sequencing. As shown in Figure 1A and B,

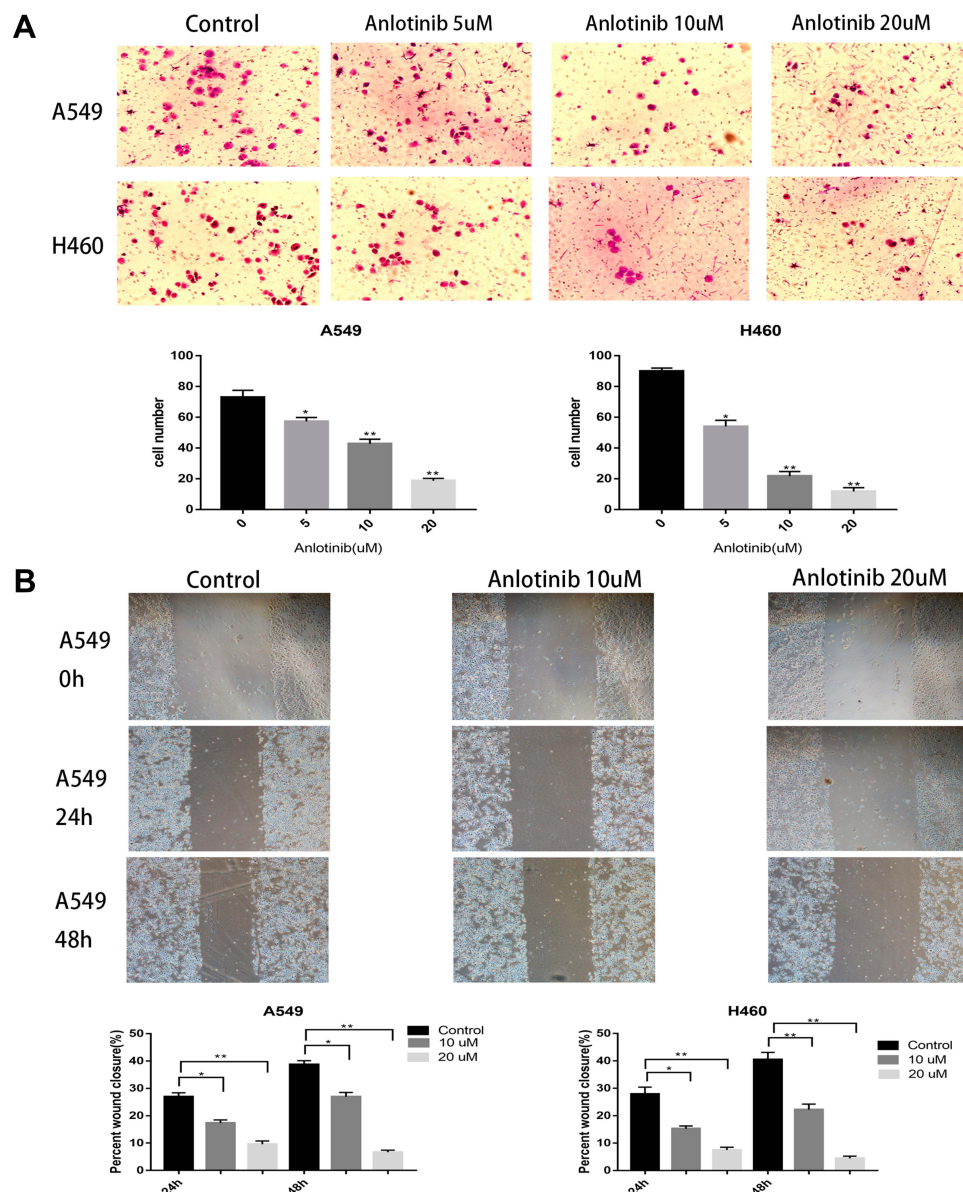


Figure 3 Anlotinib reduces metastatic potential of KRAS mutant lung cancer cells. **(A)** Transwell detection of invasive ability with Anlotinib treatment. **(B)** The Wound healing analysis of migration ability after Anlotinib treatment. * $P < 0.05$ ** $P < 0.01$

KRAS was mutated at G12S and Q61H in the A549 and H460 cells respectively. Anlotinib significantly decreased the proliferation of both cell lines in a dose-dependent manner, and the respective IC₅₀ for A549 and H460 cells were 18.84 μ M and 16.77 μ M (Figure 1C and D). Anlotinib treatment also markedly decreased the number of colonies formed by the A549 and H460 cells (Figure 1E). Furthermore, the proportion of apoptotic A549 cells increased from 12.36% to 45.05% and that of apoptotic H460 cells from 7.57% to 39.88% after Anlotinib treatment (Figure 2A). Consistent with this, Anlotinib significantly downregulated the levels of the anti-apoptotic BCL-2

protein and upregulated the pro-apoptotic BAX in A549 and H460 cells (Figure 2B).

Anlotinib Reduces Migration and Invasion of KRAS Mutant Lung Cancer Cells

To determine the possible effect of Anlotinib on the metastatic potential of KRAS mutant lung cancer cells, we performed the in vitro wound healing and transwell assays. As shown in Figure 3B, Anlotinib inhibited the migratory ability of A549 and H460 cells, and also significantly decreased the number of invasive cells (Figure 3A).

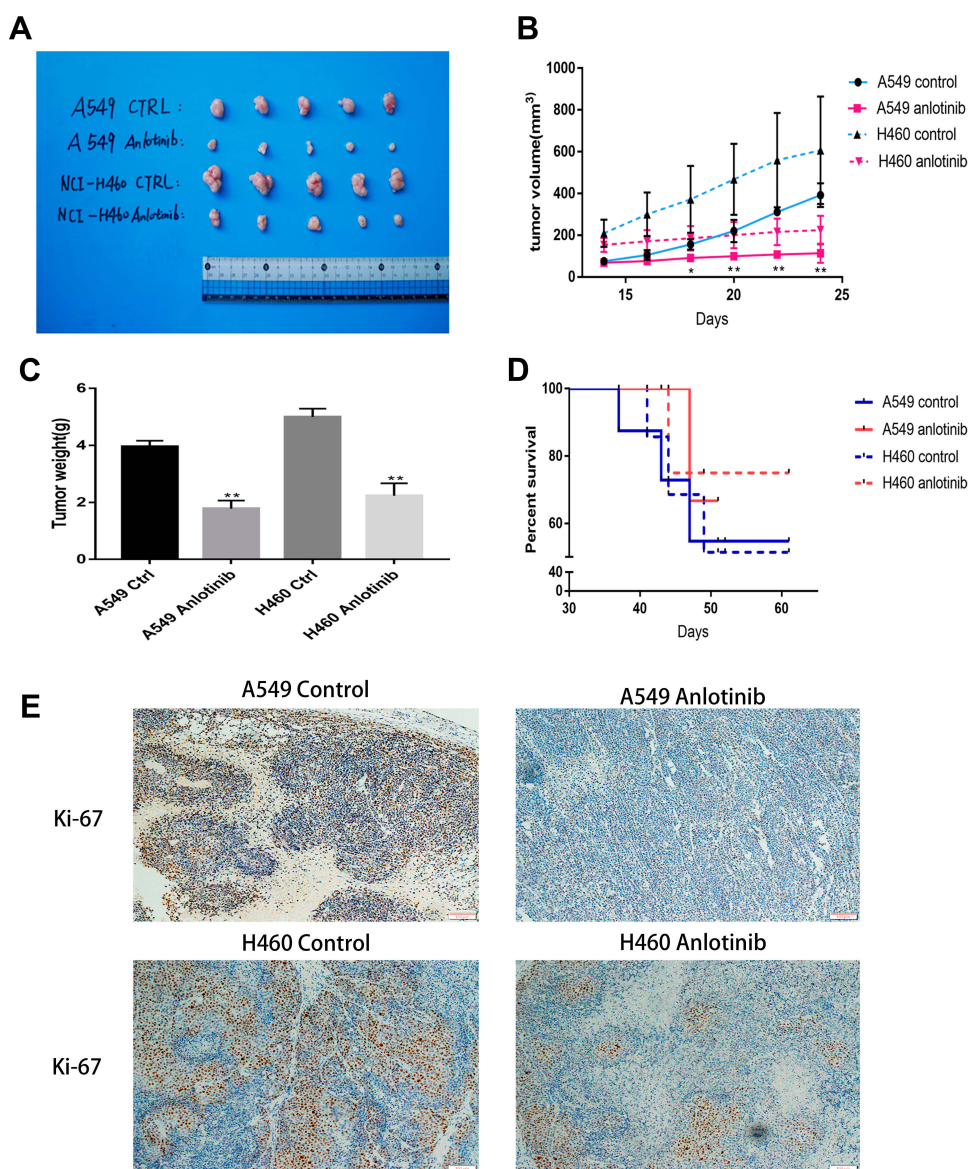


Figure 4 Anlotinib exerts anti-cancer effects in vivo. (A) Anlotinib suppresses tumor growth of NCI-H460 and A549 cells bearing mice. (B) Tumor volume of NCI-H460 and A549 cells bearing mice. (C) Tumor weight of NCI-H460 and A549 cells bearing mice. (D) Survival time of NCI-H460 and A549 cells bearing mice. (E) IHC detection of Ki67 expression in tumor tissues of NCI-H460 and A549 cells bearing mice. ** $P < 0.01$. Scale bar=100 μ m.

Anlotinib Suppresses Growth of KRAS Mutant Xenografts in Mice

To further validate the anti-cancer effects of Anlotinib *in vivo*, we established KRAS mutant lung cancer xenografts in a mouse model. As shown in [Figure 4A](#) and [B](#) and [Supplementary Figure 1A](#), Anlotinib significantly suppressed the growth of KRAS mutant tumors compared to that in the untreated controls, without affecting the body weight of mice. Consistent with this, the post-mortem tumor weight was significantly lower in the Anlotinib-treated versus the control group ([Figure 4C](#)). In addition, Anlotinib significantly decreased the expression of Ki67 in the tumor tissues

([Figure 4E](#)), and prolonged the survival of the tumor-bearing mice ([Figure 4D](#)). We also detected the extent of neo-angiogenesis in the tumor tissues by CD31 immunostaining, and observed a significant reduction in CD31+ vessel density in the treated versus the untreated group ([Supplementary Figure 1B](#)), indicating that Anlotinib also exerted its anti-cancer effects by suppressing angiogenesis.

Anlotinib Attenuates MEK/ERK Pathway in KRAS Mutant Lung Cancer Cells

To explore the possible molecular mechanism of Anlotinib action, we analyzed the expression levels of the ERK and

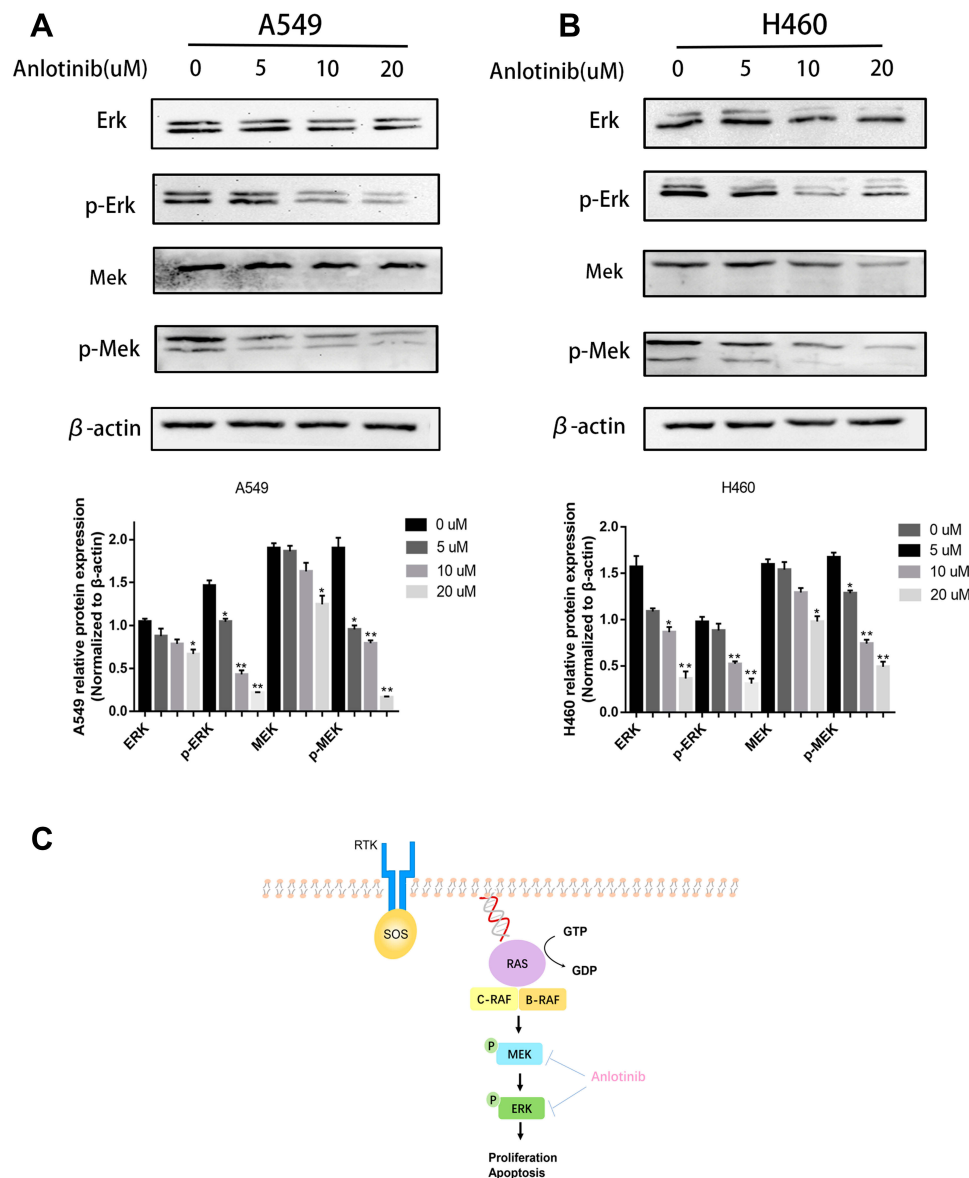


Figure 5 Anlotinib attenuates MEK/ERK pathway in KRAS mutant lung cancer cells. **(A)** Western blot analysis of ERK, p-ERK, MEK, p-MEK in A549 cells after Anlotinib treatment. **(B)** Western blot analysis of ERK, p-ERK, MEK, p-MEK in NCI-H460 cells after Anlotinib treatment. **(C)** MAPK signaling pathway in KRAS mutant cancers. * $p < 0.05$, ** $p < 0.01$.

MEK. Anlotinib downregulated both ERK and MEK, and also significantly decreased the levels of p-ERK and p-MEK in a concentration-dependent manner (Figure 5A and B). Thus, Anlotinib inhibits the growth of KRAS mutant cells by blocking MAPK signaling. The putative mechanism is outlined in Figure 5C.

Discussion

Lung cancer is associated with high incidence and mortality, and the KRAS mutations, especially in NSCLC patients, render the tumors recalcitrant to treatment.^{21–23} Anlotinib is an orally-administered multi-target TKI (RTKs). In the present study, we found that Anlotinib inhibited proliferation of KRAS mutant lung cancer cells in vitro and in vivo, and prolonged the survival of tumor-bearing mice, which is consistent with a recent clinical case report of a lung adenocarcinoma patient.¹¹ A previous study showed that Anlotinib inhibited hepatocellular carcinoma cells via apoptosis induction.²⁴ In this study as well, Anlotinib significantly induced apoptosis in the KRAS mutant lung cancer cells by downregulating the survival factor BCL-2 and upregulating the pro-apoptotic factor BAX^{25,26} in a dose-dependent manner.

Metastasis is an indicator of poor prognosis in lung cancer patients, and decreasing the metastatic potential of lung cancer cells significantly prolonged the survival of xenograft-bearing mice.^{27,28} Recently, Liu Z reported significantly higher risk of metastasis in the KRAS mutant versus wild-type lung cancer patients.²⁹ Consistent with this, Anlotinib markedly decreased the migratory and invasive abilities of KRAS mutant lung cancer cells.

The MEK/ERK signal is one of the MAPK pathways which can regulate multiple cellular processes, including proliferation, apoptosis and chemoresistance.^{30,31} Recently, Yen and Ji D reported that KRAS mutation in lung cancer cells increased the levels of phosphorylated MEK and ERK, and then promoted proliferation and suppressed apoptosis in lung cancer cells.^{6,32,33} MEK/ERK signaling plays an important role in KRAS mutant lung cancer cells. And it was found that the phosphorylation of MEK/ERK could be repressed by Anlotinib in this study.

Conclusions

Anlotinib can be included as part of the first-line treatment of KRAS mutant NSCLC patients, and needs to be validated by further functional and clinical studies.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, give final approval of version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that there is no conflict of interest.

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