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Reversal of acquired resistance to EGFR-TKI in T790M-negative patients with non-small-cell lung cancer using anlotinib

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Abstract:

Background: Treatment options for epidermal growth factor receptor (EGFR) T790M-negative patients with non-small-cell lung cancer (NSCLC) and acquired resistance

(AR) to EGFR-tyrosine kinase inhibitor (EGFR-TKI) are limited. The efficacy of EGFR-TKI and anti-angiogenic drug combination therapy in these patients is known. We investigated the effectiveness of EGFR-TKI+anlotinib combination therapy in patients with T790M-negative NSCLC.

Method: We evaluated the antitumor effects of gefitinib combined with anlotinib in gefitinib-resistant lung adenocarcinoma cells. We also investigated the treatment effect and absence of adverse events of EGFR-TKI+anlotinib therapy in 22 T790M-negative patients after EGFR-TKI treatment failure between January 2018 and August 2020.

Results: Anlotinib reversed gefitinib resistance in the gefitinib-resistant cell line, PC9/GR, by enhancing anti-proliferative and pro-apoptotic effects of gefitinib. The gefitinib+anlotinib treatment exerted a synergistic antitumor effect by downregulating the activation of VEGFR2 and downstream effectors, Akt and ERK. The EGFR-TKI+anlotinib therapy exhibited an objective response rate of 18.2% and a disease control rate of 95.5%. The median progression-free survival (PFS) was 11.53 ± 1.94 months, whereas the median overall survival was not reached. The median PFS was longer in patients exhibiting gradual progression (13.30 ± 1.69 months) than in patients with dramatic progression (8.60 ± 5.39 months, $p = 0.041$). One Grade 3 adverse event was noted (diarrhea, $n = 2$, 9.1%), and Grade 4 or 5 adverse events were absent.

Conclusion: EGFR-TKI combined with anlotinib demonstrated powerful antitumor activity in vitro and excellent treatment effect in T790M-negative NSCLC patients after AR.

Keywords: Non-small-cell lung cancer; EGFR; VEGFR; Gefitinib; Anlotinib.

Declarations:

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Conflict of interest:

The authors declare no potential conflicts of interest.

Availability of data and material:

Please contact authors for data requests.

Authors' contributions:

Chen Zhang: Conceptualization, Methodology, Formal analysis, Writing-Original Draft, Funding acquisition. **Honggang Cao:** Conceptualization, Methodology, Formal analysis. **Shidai Jin:** Resources, Data Curation. **Wen Gao:** Resources, Data Curation.

Chenjun Huang: Conceptualization, Methodology, Supervision. **Renhua Guo:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition.

Ethics approval:

Not applicable.

Consent for publication:

Not applicable.

Abbreviations:

EGFR	epidermal growth factor receptor
NSCLC	non–small-cell lung cancer
AR	acquired resistance
TKI	tyrosine kinase inhibitor
PFS	progression-free survival
VEGFR	vascular endothelial growth factor receptor
OS	overall survival
ORR	objective response rate
DCR	disease control rate

Background

Lung cancer is one of the most fatal cancer worldwide, and non–small-cell lung cancer (NSCLC) is the most common histological type accounting for up to 85% of lung cancer cases^[1, 2]. The investigation of lung cancer pathogenesis and identification of various therapeutic molecular targets has increased the significance of targeted therapy in NSCLC. The most widely used targeted agents, epidermal growth factor receptor–tyrosine kinase inhibitor (EGFR–TKI), significantly prolonged the survival in EGFR-mutant patients with NSCLC^[3, 4]. However, the clinical benefits of EGFR–TKI therapies are challenged by acquired resistance (AR). EGFR-T790M mutation is the most common mechanism of AR^[5]. Third-generation EGFR-TKI such as osimertinib have been reported to produce promising responses in T790M-positive patients^[6]. However, the treatment options for T790M-negative patients are limited.

Vascular endothelial growth factor receptor (VEGFR) is another significant target for the treatment of NSCLC. Molecular research exhibits that VEGFR and EGFR share common signaling pathways such as PI3K/Akt and mitogen-activated protein kinase signaling pathways. EGFR inhibition can downregulate VEGF expression by hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms^[7]. Locally secreted VEGF promotes angiogenesis in the tumor microenvironment and is involved in EGFR–TKI resistance^[8]. Based on these findings, we speculated that the dual

targeting of EGFR and VEGFR may be theoretically effective in patients with acquired EGFR–TKI resistance. Numerous recent clinical trials support our hypothesis. Afatinib plus bevacizumab (a potent monoclonal antibody targeting vascular endothelial growth factor A) have reported positive treatment effect in patients with NSCLC after AR to EGFR–TKI^[9]. Apatinib, a small-molecule TKI that blocks VEGFR2, has also been reported to enhance the antitumor activity of EGFR–TKI in NSCLC with AR^[10].

Anlotinib is a novel oral multi-targeted small-molecular TKI approved for the treatment of third-line advanced NSCLC. Anlotinib mainly exerts its anti-angiogenic and broad-spectrum antitumor effects by the highly potent and specific suppression of VEGFR2^[11]. A subgroup analysis of the ALTER0303 trial demonstrated that patients previously treated with gefitinib exhibited better progression-free survival (PFS) and overall survival (OS) after treatment with anlotinib^[12]. However, a recent study conversely reported that anlotinib exhibited an inferior treatment effect compared with chemotherapy in T790M-negative patients with NSCLC after first-line EGFR–TKI treatment failure^[13].

Therefore, the present study investigated whether combined anlotinib and gefitinib treatment could reverse gefitinib resistance *in vitro* and whether T790M-negative patients with NSCLC could benefit from this combination therapy after EGFR–TKI treatment failure.

Method

Cell lines and reagents

Human NSCLC cells, PC9, were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The gefitinib-resistant cells, PC9/GR, were provided by Shanghai Pulmonary Hospital. All the cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/mL and streptomycin 100 mg/mL).

Gefitinib (CAS No. 184475-35-2) and anlotinib (CAS No. 1360460-82-7) were purchased from Selleck chemicals LLC (Houston, TX, USA). All chemicals were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiments. Antibodies against

EGFR, p-EGFR, VEGFR2, p-VEGFR2, ERK1/2, p-ERK1/2, Akt, p-Akt, and cleaved-caspase 3 were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell proliferation assays

Cell viability assay

Cell viability was measured by CCK8 assay (cell counting kit-8, Selleck, Shanghai, China). PC9 and PC9/GR cells were plated in 96-well plates at a density of 3×10^3 /well and incubated overnight. Subsequently, the cells were exposed to increasing concentrations of gefitinib (0.01, 0.1, 1.0, 2.0, 5.0, and 10.0 μM), anlotinib (1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μM), or a gefitinib–anlotinib combination (gefitinib: 0.01, 0.1, 1.0, 2.0, 5.0, and 10.0 μM ; with anlotinib 2.0 μM) for 72 h. Then, 10 μL of CCK8 was added into each well and incubated for 1 h. The absorbance (optical density, OD) at 450 nm was detected using a microplate reader (Tecan, Mechelen, Belgium). Cell viability was calculated as: $\text{Cell viability} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%$. IC50 value was calculated by GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Experiments were repeated in triplicate, and an average was obtained. The combination index for the combination of gefitinib and anlotinib was calculated using CompuSyn software.

5-Ethynyl-20-deoxyuridine cell proliferation assay

The PC9/GR cells were cultured in 12-well plates at 1×10^5 cells/well overnight. Subsequently, the cells were exposed to DMSO, 0.1 μM gefitinib, 2 μM anlotinib, or a combination of 0.1 μM gefitinib and 2 μM anlotinib for 48 h. The Ethynyl-20-Deoxyuridine (EdU) incorporation assay was performed using an EdU assay kit (C0071S, Beyotime, Shanghai, China) according to the manufacturer instructions. 10 μM EdU was added to each well and allowed 2 h for incorporation. After treatment with 4% paraformaldehyde and 0.3% Triton X-100, the cells were stained with anti-EdU click additive solution. Hoechst 33342 was used to label the cell nuclei. The percentage of EdU-positive cells was calculated after fluorescence microscopy analysis. Three fields of view were randomly assessed for each treatment group.

Colony forming assay

PC9/GR cells were seeded in 6-well plates at 500 cells/well overnight. They were

then treated with DMSO, 0.1 μM gefitinib, 2 μM anlotinib, or a combination of 0.1 μM gefitinib and 2 μM anlotinib for 24 h. Complete medium was replaced every 3 days. After 10 days, the colonies were fixed with methanol and stained with a 0.1% crystal violet (Sigma, St. Louis, MO, USA). Total colonies with diameter > 0.5 mm were counted. Experiments were repeated in triplicate, and an average was obtained.

Cell apoptosis detection

PC9/GR cells were seeded overnight in 6-well plates at 1×10^5 cells/well. They were then treated with DMSO, 0.1 μM gefitinib, 2 μM anlotinib, or a combination of 0.1 μM gefitinib and 2 μM anlotinib. Cell apoptosis detection was performed using Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (40302, Yeasen, Shanghai, China) according to the manufacturer instructions. The treated cells were briefly harvested and washed with phosphate-buffered saline, followed by suspension in 100 μL of Annexin V binding buffer. Then, 5 μL of Annexin V-FITC and 10 μL of PI were added for incubation in the dark. Samples were then analyzed using a flow cytometer (FACScan, BD Biosciences). Experiments were performed in triplicate, and an average was obtained.

Protein expression by Western blotting

The total cellular protein lysates of PC9 and PC9/GR cells were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, USA). The membranes were incubated with specific antibodies against EGFR, p-EGFR, VEGFR2, p-VEGFR2, ERK1/2, p-ERK1/2, Akt, p-Akt, and cleaved-caspase 3 overnight at 4°C. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for EGFR, p-EGFR, VEGFR2, p-VEGFR2, ERK1/2, p-ERK1/2, Akt, and p-Akt, whereas β -actin was used as an internal control for cleaved-caspase 3. The immunoreactive bands were visualized with enhanced chemiluminescence (ECL) using an ECL detection system. The band densities were quantified using ImageJ (NIH). Experiments were performed in triplicate, and an average was obtained.

Clinical data collection

Study population

The present retrospective study was conducted in the department of Medical Oncology at the First Affiliated Hospital of Nanjing Medical University between January 2018 and August 2020. This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Patients with cytologically or histologically confirmed NSCLC with EGFR-sensitive mutation, exhibiting AR to first- or second-generation EGFR-TKI [according to the criteria by Jackman et al^[14]], confirmed T790M negative by rebiopsy after AR, and those on a combination therapy of EGFR-TKI and anlotinib (Chia Tai Tianqing Pharmaceutical Group Co., Ltd., Jiangsu, China) as subsequent therapy were included in the study. As for rebiopsy, after the patients tested EGFR-positive by first-time biopsy and exhibited resistance to targeted therapy, they were rebiopsied to evaluate T790M mutation. Only T790M-negative patients were included in the study. Patients lost to follow-up or those with a follow-up period (between the first treatment of anlotinib and the latest follow-up) of less than 2 months were excluded from the study. EGFR-TKI was continued, and anlotinib (one cycle of 10 mg p.o. daily for 14 days, discontinued for 7 days, and repeated every 21 days) was added until disease progression or intolerable toxicity. The dosage adjustments (increase or decrease) were decided by the attending physician according to clinical response and adverse events. The medical records of these patients were collected to study related characteristics such as age, sex, histologic type, EGFR mutation type, and prior EGFR-TKI treatment.

Assessment of treatment effect and adverse events

Tumor assessment was performed using radiographical data according to the Response Evaluation Criteria in Solid Tumor (RECIST), version 1.1. The modes of EGFR-TKI treatment failure were divided into dramatic progression (disease control lasting ≥ 3 months; rapid increment of tumor burden compared with the previous assessment; symptom scored 2), gradual progression (disease control lasting ≥ 6 months; minor increment of tumor burden compared with the previous assessment; symptom scored ≤ 1), and local progression (disease control lasting ≥ 3 months; solitary extracranial or intracranial progression; symptom scored ≤ 1) according to the criteria by Yang et al^[15]. PFS was defined as the time from the initiation of combination therapy

till the first observed progression. OS was defined as the time from the initiation of combination therapy till death. The adverse events were assessed according to the Common Terminology Criteria for Adverse Events (CTCAE, version 5.0).

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, USA) and GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Continuous variables were presented as means \pm standard deviations, or as median along with the minimum–maximum range, if not normally distributed. Categorical variables were described as n (%). Student's t test was used for continuous variables. PFS and OS were estimated using the Kaplan–Meier method. Subgroup analyses were performed using the EGFR mutation type and EGFR–TKI failure mode. The PFS between groups was compared using the log-rank test. A P value of <0.05 was considered statistically significant.

Results

Resensitization of PC9/GR cells to gefitinib by anlotinib

To demonstrate the resistance of PC9/GR cells to gefitinib, we treated the PC9 and PC9/GR cells with gefitinib for 72 h and detected the cell viability through the CCK8 assay. The values of the half maximal inhibitory concentration (IC₅₀) in PC9/GR cells [IC₅₀: 4.47 μ M, 95% confidence interval (CI): 3.49–5.73 μ M] were approximately 9-fold higher than those in the gefitinib-sensitive cell line PC9 (IC₅₀: 0.49 μ M, 95% CI: 0.09–1.19 μ M) (Figure 1A).

As a multitargeted small-molecular TKI, anlotinib induced similar inhibitory effects on PC9 and PC9/GR cell viability (Figure 1B; IC₅₀: 2.53 μ M, 95% CI: 1.88–3.39 μ M in PC9 vs IC₅₀: 2.51 μ M, 95% CI: 1.93–3.26 μ M in PC9/GR). Moderate inhibitory effects were exhibited on both cell lines (about 41.6% on PC9 cells and 48.5% on PC9/GR cells) by 2 μ M anlotinib. Therefore, it was selected for the combination experiments. Gefitinib significantly inhibited cell growth in PC9/GR cells when used in combination with 2 μ M anlotinib (Figure 1C; IC₅₀ of gefitinib: 0.05 μ M, 95% CI: 0.01–0.68 μ M).

The combination index was calculated using the Chou–Talalay method to determine whether the combined effect was synergistic. The calculated combination indices ranged from 0.3 to 0.7 (Figure 1D), which indicated a synergistic effect. The PC9/GR cells with a combination index value of 0.6 for 0.1 μM gefitinib combined with 2 μM anlotinib (Figure 1D, E) were selected for further exploration.

Enhancement of the anti-proliferative effect of gefitinib on PC9/GR cells by anlotinib

To determine the anti-proliferation effect of the combination treatment of anlotinib and gefitinib on PC9/GR cells, the EdU incorporation assay was performed to evaluate the activity of DNA synthesis and cell proliferation. The number of EdU-positive cells in the combination treatment group was lesser than that in the isolated 0.1 μM gefitinib treatment group (Figure 2A, B; $P < 0.001$).

The colony formation assay was then conducted to reflect the proliferative capacity of single cells. The number of colonies of PC9/GR cells were significantly reduced after combined treatments of 0.1 μM gefitinib and 2 μM anlotinib compared with isolated gefitinib treatment (Figure 2C, D; $P < 0.001$). Thus, these results demonstrated that gefitinib combined with anlotinib synergistically inhibited the proliferation of PC9/GR cells.

Enhancement of the pro-apoptotic effect of gefitinib on PC9/GR cells using anlotinib

Flow cytometric analysis was performed using Annexin V-FITC and PI double staining to evaluate the effect of the combination on PC9/GR cells apoptosis. After treatment with 0.1 μM gefitinib alone or a combination of 0.1 μM gefitinib and 2 μM anlotinib for 48 h, we detected a significantly higher total apoptosis rate ($62.3\% \pm 27.9\%$) in the PC9/GR cells of the combination group than in the gefitinib group ($16.1\% \pm 4.1\%$; $P = 0.047$; Figure 3A and 3C). Furthermore, the protein expression of cleaved-caspase 3 was detected as a marker for apoptosis, and the results exhibited the upregulation of protein expression level in the combination group relative to the gefitinib group (Figure 3B and 3D; $P = 0.003$). These results indicated that the pro-apoptotic ability of gefitinib was enhanced by anlotinib.

Gefitinib and anlotinib synergistically inhibited the Akt and ERK signaling pathways.

The effect of gefitinib and/or anlotinib on PC9 and PC9/GR cells was further studied to investigate the mechanisms of the drug combination. Protein expression levels of EGFR, VEGFR2, and their downstream signaling were determined by Western blotting. After the 48-h treatment, 0.1 μ M gefitinib significantly downregulated the expression of phosphorylated EGFR (p-EGFR) in both PC9 and PC9/GR cells (Figure 4 and Supplementary Figure 1). However, decreased phosphorylated ERK (p-ERK) and phosphorylated Akt (p-Akt) expression was observed only in PC9 cells. The p-ERK, p-Akt, and phosphorylated VEGFR2 (p-VEGFR2) expression levels in PC9/GR cells combined with 2 μ M anlotinib were dramatically decreased. Thus, we can conclude that anlotinib reversed gefitinib resistance in PC9/GR cells through the inhibition of VEGFR2 phosphorylation and downregulation of ERK and Akt signaling (Supplementary Figure 2).

Reversal of the EGFR–TKI resistance in patients by anlotinib

The effect of anlotinib was further validated on reversal of the EGFR–TKI resistance in patients with NSCLC. Of the 25 patients included between January 2018 and August 2020, 1 patient was lost to follow-up, and 2 patients had a follow-up time < 2 months. Eventually, 22 patients were included in the final analysis. Baseline characteristics of these patients are presented in Table 1. In these patients, L858R (n = 11, 50.0%) was the most frequently observed EGFR mutation. Deletion in exon 19 was observed in 8 (36.4%) patients, and 3 (13.6%) patients had other mutations. Most patients (n = 20, 90.9%) received prior first-generation EGFR–TKI treatment such as gefitinib (n = 17, 77.3%), icotinib (n = 2, 9.1%), and erlotinib (n = 1, 4.5%), whereas 2 of the 3 patients (9.1%) with rare mutations in EGFR received prior afatinib treatment. EGFR–TKI failure in these patients was divided into the following two modes: 3 patients with dramatic progression (13.6%) and 19 with gradual progression (86.4%). After the imaging demonstrated disease progression, anlotinib was administered in combination with EGFR–TKI mostly at a starting dose of 10 mg p.o. daily. The dosage of only one patient was decreased to 8 mg p.o. daily because of diarrhea, whereas no

patient required dosage increase.

A confirmed partial response was observed in 4 (18.2%) patients after combination therapy, whereas 17 (77.3%) patients exhibited stable disease (Figure 5A, B). Unfortunately, 1 patient (4.5%) was insensitive to this combination strategy and exhibited disease progression in his first assessment, resulting in an objective response rate (ORR) of 18.2% and disease control rate (DCR) of 95.5%. The median follow-up time was 8.39 months. The median PFS was 11.53 ± 1.94 months (Figure 5C), whereas the median OS was not reached.

Subgroup analysis was performed according to type of EGFR mutations. The median PFS appeared to be longer in the Del-19 group (not reached) than in the L858R (8.60 ± 1.79 months) and rare mutation (9.63 ± 6.23 months) groups, although the difference was not statistically significant ($P = 0.186$, Figure 5D). Subgroup analysis according to the mode of EGFR-TKI failure was then performed. The median PFS in patients with gradual progression (13.30 ± 1.69 months) was much longer than that in patients with dramatic progression (8.60 ± 5.39 months, $p = 0.041$; Figure 5 E).

The adverse events are presented in Table 2. The most common adverse events were rash ($n = 9$, 40.9%) and diarrhea ($n = 6$, 27.3%) related to EGFR-TKI followed by transaminase elevation ($n = 4$, 18.2%). Only one adverse event was graded as Grade 3 (diarrhea, $n = 2$, 9.1%), and no adverse event was graded as Grade 4 or 5. Other adverse events such as paronychia, thrombosis, bleeding, proteinuria, and leukopenia were rarely seen.

Discussion

AR development is a major challenge in the EGFR-TKI treatment of NSCLC. More than 50% of all AR to EGFR-TKI is attributed to the development of the EGFR T790M mutation^[5]. T790M-positive patients often respond to the third-generation EGFR-TKI, exhibiting better prognosis than T790M-negative patients^[6]. Thus, an effective therapeutic strategy must be investigated for these T790M-negative patients. The present study observed that gefitinib combined with anlotinib exhibited a significant antitumor effect on gefitinib-resistant lung adenocarcinoma cells in vitro and

that the combination therapy of EGFR-TKI and anlotinib demonstrated promising treatment effects and negligible adverse events in T790M-negative patients with NSCLC. To the best of our knowledge, this is the first study to evaluate the effect of the combination therapy of EGFR-TKI and anlotinib on T790M-negative patients after EGFR-TKI treatment failure.

Anlotinib is a multi-targeted small-molecular TKI that inhibits a group of kinases such as VEGFR1/2/3, c-Kit, and PDGFR β ^[11]. In China, this drug has been approved as third-line treatment of locally advanced or metastatic cases of NSCLC with progression or recurrence^[16]. The ALTER 0303 clinical trial demonstrated favorable outcomes in patients with advanced NSCLC receiving anlotinib as a third-line or further therapy^[17]. Molecular research has shown that by targeting VEGFR2, anlotinib plays anti-angiogenesis and antitumor roles in NSCLC^[11]. The present study observed that anlotinib induced sensitivity of the gefitinib-resistant lung adenocarcinoma cell line PC9/GR to gefitinib by enhancing the anti-proliferative and pro-apoptotic effects of gefitinib. Due to the tight connection between the EGFR and VEGFR signaling, we further performed Western blotting to detect protein expression levels of EGFR, VEGFR2 and their downstream signaling. The activation of VEGFR2 and the phosphorylation of the downstream effectors, Akt and ERK, were significantly downregulated in the combination group. These results indicated that the combination treatment of anlotinib and gefitinib overcame the EGFR-TKI resistance, possibly by inhibiting the activation of VEGFR2 and the downstream Akt and ERK signaling transduction pathways.

Several studies have demonstrated that EGFR-TKI combined with anti-angiogenic drugs such as bevacizumab^[9], apatinib^[18], and ramucirumab^[19] exhibit encouraging clinical activity in patients with NSCLC. However, there are currently no similar reports providing clinical outcomes for the combination of EGFR-TKI and anlotinib. In the present study, we retrospectively reviewed the medical records of 22 eligible patients with NSCLC who received the combination therapy of EGFR-TKI and anlotinib after EGFR-TKI treatment failure. The combination therapy exerted favorable effects on T790M-negative patients after EGFR-TKI resistance, with an ORR of 18.2% and a

DCR of 95.5%. Short-term prognosis was also observed to be superior with the median PFS of 11.53 ± 1.94 months. The adverse events data suggested this regimen was well tolerated with no Grade 4 or 5 adverse events reported. Thus, the combination of EGFR-TKI and anlotinib could be considered as a therapeutic salvage option for the T790M-negative population.

The treatment effect of EGFR-TKI varies among different EGFR mutations. Therefore, we performed subgroup analysis according to the type of EGFR mutations. The estimated median PFS was longer in the Del-19 group (not reached) than in the L858R (8.60 ± 1.79 months) and rare mutation (9.63 ± 6.23 months) groups, although this difference was not statistically significant ($P = 0.186$). A larger sample size could demonstrate more credible results.

Yang et al divided the diversity of EGFR-TKI failure into three modes according to specific criteria derived from clinical factors, namely dramatic progression, gradual progression, and local progression^[15]. In our study, 19 patients (86.4%) experienced gradual progression, whereas the other 3 patients (13.6%) experienced dramatic progression. These patients received a combination therapy of EGFR-TKI and anlotinib due to refusal to chemotherapy. We further performed subgroup analysis according to EGFR-TKI failure mode and discovered that patients exhibiting gradual progression (median PFS, 13.30 ± 1.69 months) are more likely to benefit from this combination strategy than the those exhibiting dramatic progression (8.60 ± 5.39 months, $P = 0.041$). This finding is concurrent with Yang et al's study^[15], which reported that patients in the dramatic progression group demonstrated a better survival when switching to chemotherapeutic regimens, whereas continuation of EGFR-TKI achieved significantly longer OS in the patients exhibiting gradual progression. Our findings are consistent with a previous subgroup analysis in the ALTER 0303 trial, which reported that patients could benefit from anlotinib after prior gefitinib therapy^[12]. In contrast, another recent research noted that anlotinib is less effective than chemotherapy in T790M-negative patients after first-line EGFR-TKI treatment^[13]. The lack of data regarding EGFR-TKI failure modes in these studies may explain this difference in results. The ABC clinical trial of the combination of afatinib and bevacizumab after AR

to EGFR-TKI in EGFR-mutant NSCLC exhibited a median PFS of 6.3 months^[9]. A retrospective analysis of EGFR-TKI combined with apatinib after EGFR-TKI failure exhibited a median PFS of 4.6 months^[10]. The present study suggested a much longer median PFS of 11.5 months, which indicated a strong synergistic effect of the EGFR-TKI and anlotinib combination therapy.

Despite the significant findings, the present study has several limitations. It was a single-center retrospective study with a small sample size. Prospective multicenter studies with a larger sample size are required to further strengthen our finding. Moreover, we only included patients experiencing first- and second-generation EGFR-TKI treatment failure. Unexpectedly, patients suffering from third-generation EGFR-TKI treatment failure also respond to the EGFR-TKI and anlotinib combination therapy in our clinical practice. Thus, further studies on the antitumor effects of the combination of anlotinib and third-generation EGFR-TKI in T790M-positive patients would be beneficial. Furthermore, the retrospective design of the study prevented the intensive investigation of the mechanism of reversing EGFR-TKI resistance by combination therapy. We demonstrated that the combination treatment of anlotinib and gefitinib inhibited the activation of VEGFR2 and the downstream Akt and ERK signaling transduction pathways *in vitro*. However, more in-depth studies for clarifying the molecular mechanism are still required.

Conclusion

Thus, we demonstrated that the gefitinib-anlotinib combination overcame gefitinib AR *in vitro* and the combination strategy of EGFR-TKI and anlotinib exhibited superior treatment effects and negligible adverse events in T790M-negative patients with NSCLC after EGFR-TKI treatment failure, especially in patients experiencing gradual progression. Our research provides a new therapeutic option for this subgroup of patients.

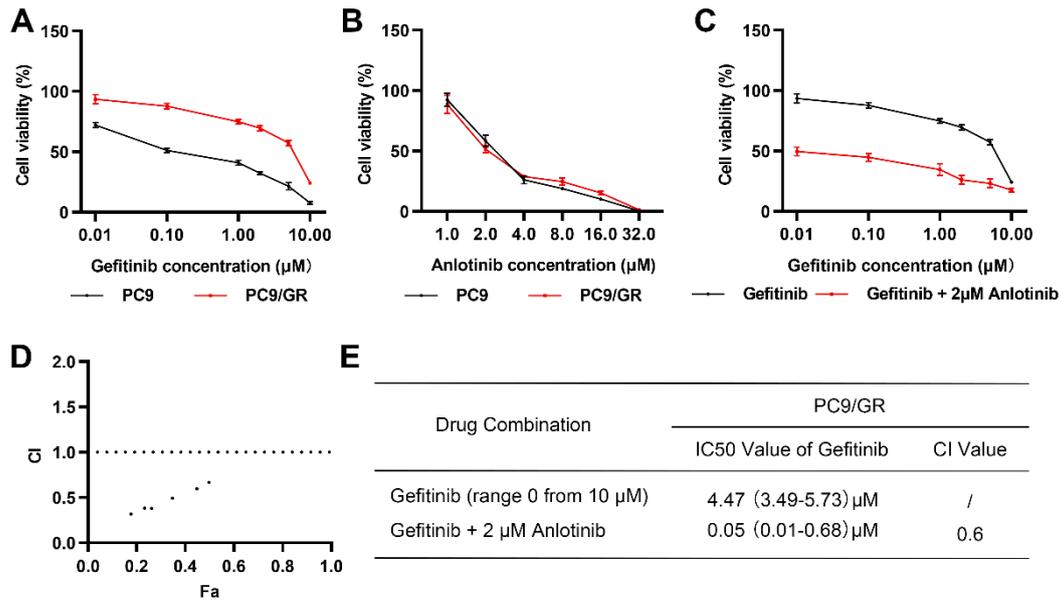


Figure 1. Anlotinib resensitized gefitinib-resistant PC9/GR cells to gefitinib. Parental lung adenocarcinoma PC9 cells and gefitinib-resistant PC9/GR cells were treated with indicated concentrations of gefitinib (A), anlotinib (B), or gefitinib plus anlotinib (C) for 72 h. Cell viability was measured by the CCK8 assay. D, The combination index of gefitinib plus anlotinib was calculated using CompuSyn software. E, Half maximal inhibitory concentration (IC₅₀) and combination index (CI) values of gefitinib alone or combined with anlotinib in PC9/GR cells.

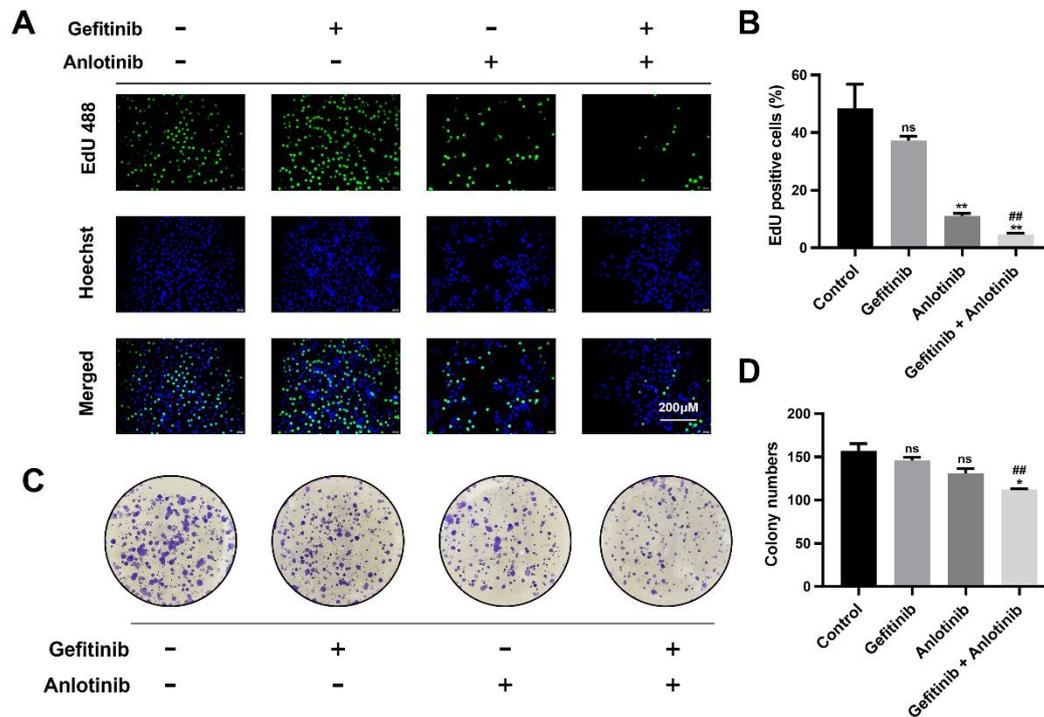


Figure 2. Treatment with gefitinib plus anlotinib synergistically inhibited proliferation of PC9/GR cells. **A** and **B**, EdU proliferation assays were performed 48 h after treatment with gefitinib, anlotinib, and gefitinib plus anlotinib. **C** and **D**, Colony-formation assays were performed to analyze the colony-formation efficiency of PC9/GR cells in the different treatment groups. Results exhibited as mean \pm SD of three independent experiments performed in triplicates. Significance levels determined using the *t* test are indicated (ns: not significant compared with the control group. * $P < 0.01$, ** $P < 0.001$ compared with the control group. # $P < 0.01$, ## $P < 0.001$ compared with the gefitinib group).

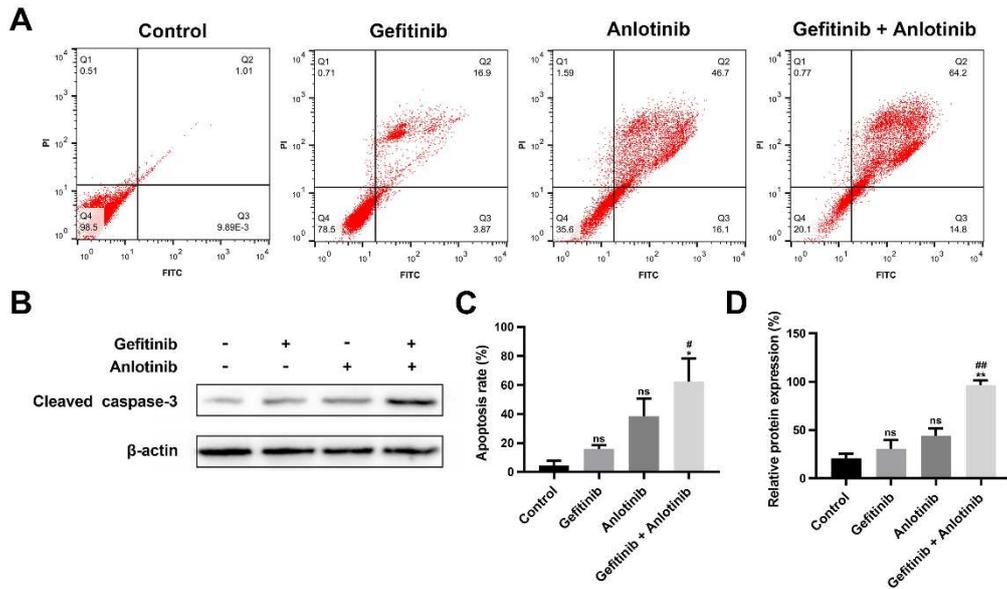


Figure 3. Treatment with gefitinib plus anlotinib synergistically promoted apoptosis of PC9/GR cells. **A** and **C**, After treatment with gefitinib, anlotinib, and gefinib plus anlotinib for 48 h, annexin V-FITC/PI staining was used to determine the apoptosis rate of PC9/GR cells. **B** and **D**, The apoptotic marker protein, cleaved-caspase 3, was analyzed by western blot analysis. Significance levels determined by the *t* test are indicated (ns: not significant compared with the control group. * $P < 0.05$, ** $P < 0.01$ compared with the control group. # $P < 0.05$, ## $P < 0.01$ compared with the gefitinib group).

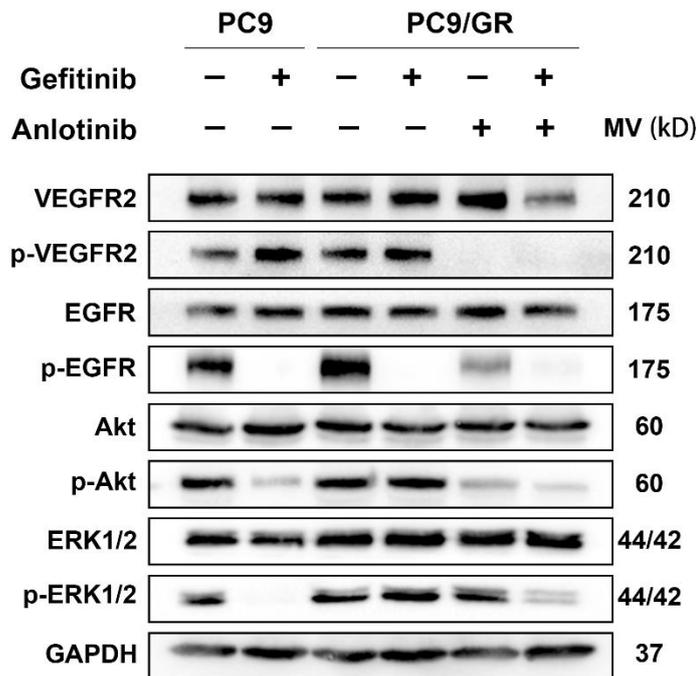


Figure 4. Gefitinib and anlotinib synergistically inhibited the Akt and ERK signaling pathways. After 48-h treatment with gefitinib, anlotinib, and gefinib plus anlotinib, the key proteins involved in the EGFR and VEGFR2 downstream signaling pathways such as VEGFR2, p-VEGFR2, EGFR, p-EGFR, Akt, p-Akt, ERK1/2, and p-ERK1/2, were analyzed by Western blot analysis.

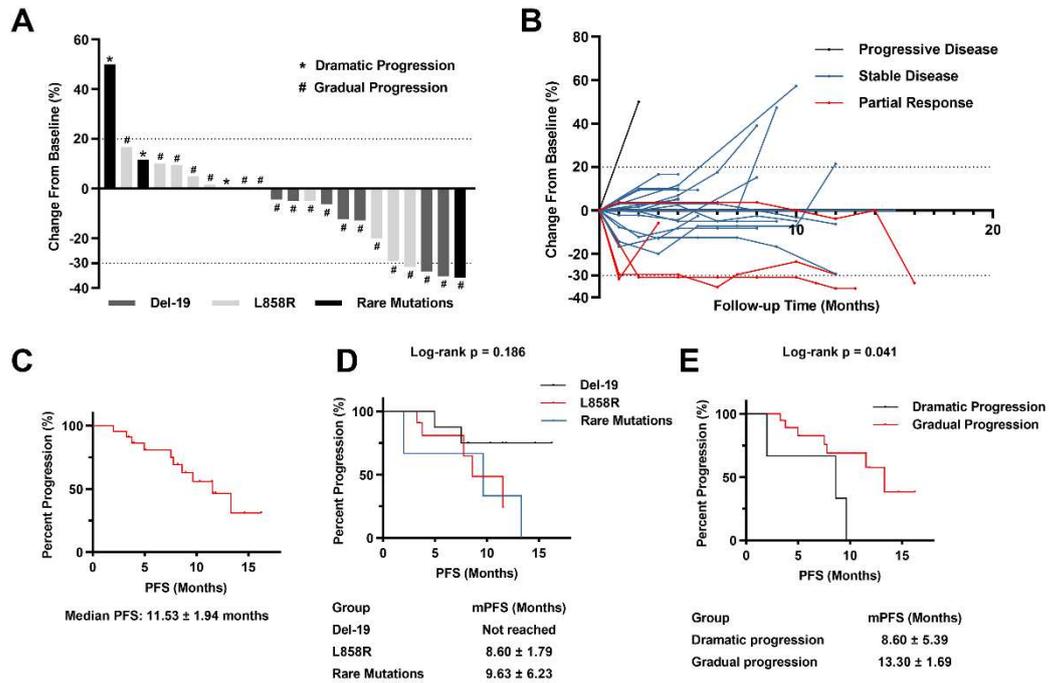


Figure 5. Anlotinib reversed the EGFR-TKI resistance in patients. **A**, The maximal tumor shrinkage of patients receiving EGFR-TKI and anlotinib therapy. **B**, The changes of tumor burden in individual patients after combination therapy. **C**, Kaplan-Meier PFS curve. **D**, Kaplan-Meier PFS curves stratification based on EGFR mutation types. **E**, Kaplan-Meier PFS curves stratification based on prior EGFR-TKI failure modes.

Table 1. Baseline characteristics (N=22).

Characteristic	Value
Age, median (range), year	68.5 (37–87)
Sex, n (%)	
Male	12 (54.5)
Female	10 (45.5)
Histology, n (%)	
Adenocarcinoma	19 (86.4)
Squamous cell carcinoma	1 (4.5)
Others	2 (9.1)
EGFR mutation, n (%)	
Exon 19 deletion	8 (36.4)
Exon 21 L858R	11 (50.0)
Rare mutations	3 (13.6)
First prior EGFR–TKI, n (%)	
Gefitinib	17 (77.3)
Erlotinib	1 (4.5)
Icotinib	2 (9.1)
Afatinib	2 (9.1)
Prior EGFR–TKI failure mode, n (%)	
Dramatic progression	3 (13.6)
Gradual progression	19 (86.4)
Follow-up period, median (range), month	8.39 (2.00-16.23)

EGFR=epidermal growth factor receptor; TKI=tyrosine kinase inhibitor. Continuous variables were given as median and the minimum/maximum range. Categorical variables were described as n (%).

Table 2. Treatment-related adverse events.

Adverse event	Grade 1, No.	Grade 2, No.	Grade 3, No.	All grades, %
Rash	5	4	0	40.9
Diarrhea	3	1	2	27.3
Transaminase elevation	3	1	0	18.2
Bleeding	0	3	0	13.6
Proteinuria	1	1	0	9.1
Paronychia	0	1	0	4.5
Thrombosis	0	1	0	4.5
Leukopenia	0	1	0	4.5

There were no grade 4/5 adverse events.

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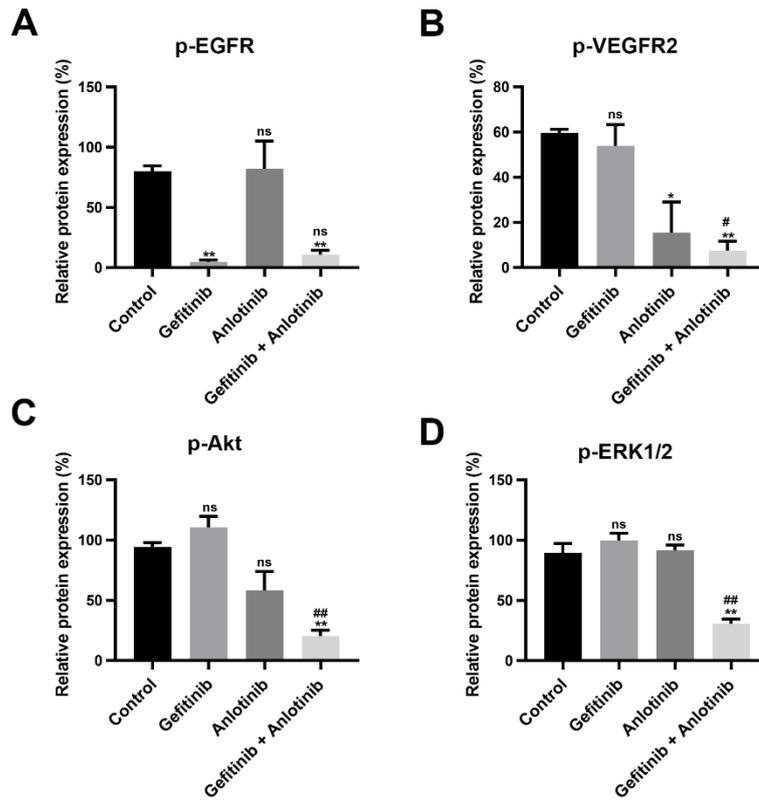
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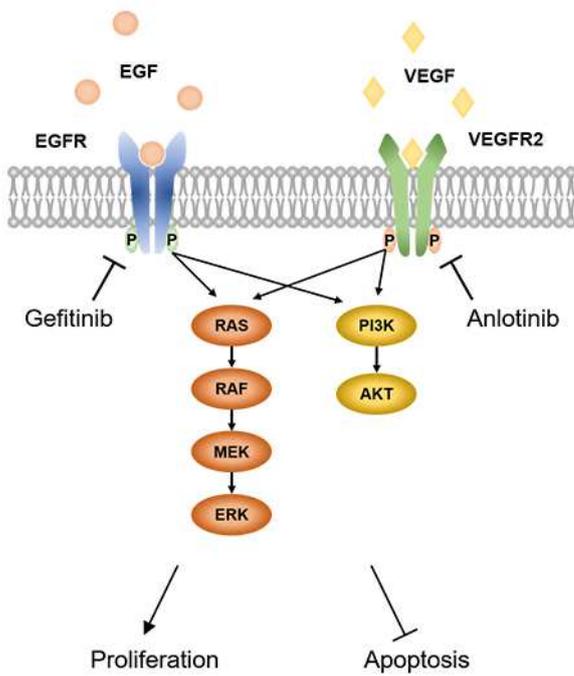
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Supplementary Figure 1. Effect of gefitinib and anlotinib on key signal transduction proteins in PC9/GR cells. PC9/GR cells were treated with gefitinib, anlotinib, or gefinib plus anlotinib for 48 h, Western blot analysis was performed to detect the expression of key signal transduction proteins. Significance levels determined by the *t* test are indicated (ns: not significant compared with the control group. * $P < 0.05$, ** $P < 0.01$ compared with the control group. # $P < 0.05$, #### $P < 0.01$ compared with the gefitinib group).



Supplementary Figure 2. Diagram of the possible mechanism of reversing gefitinib resistance by anlotinib. Gefitinib and anlotinib synergistically promoted PC9/GR cells proliferation and inhibited PC9/GR cells apoptosis through the inhibition of EGFR phosphorylation, VEGFR2 phosphorylation and the downregulation of ERK and Akt signaling.

Figures

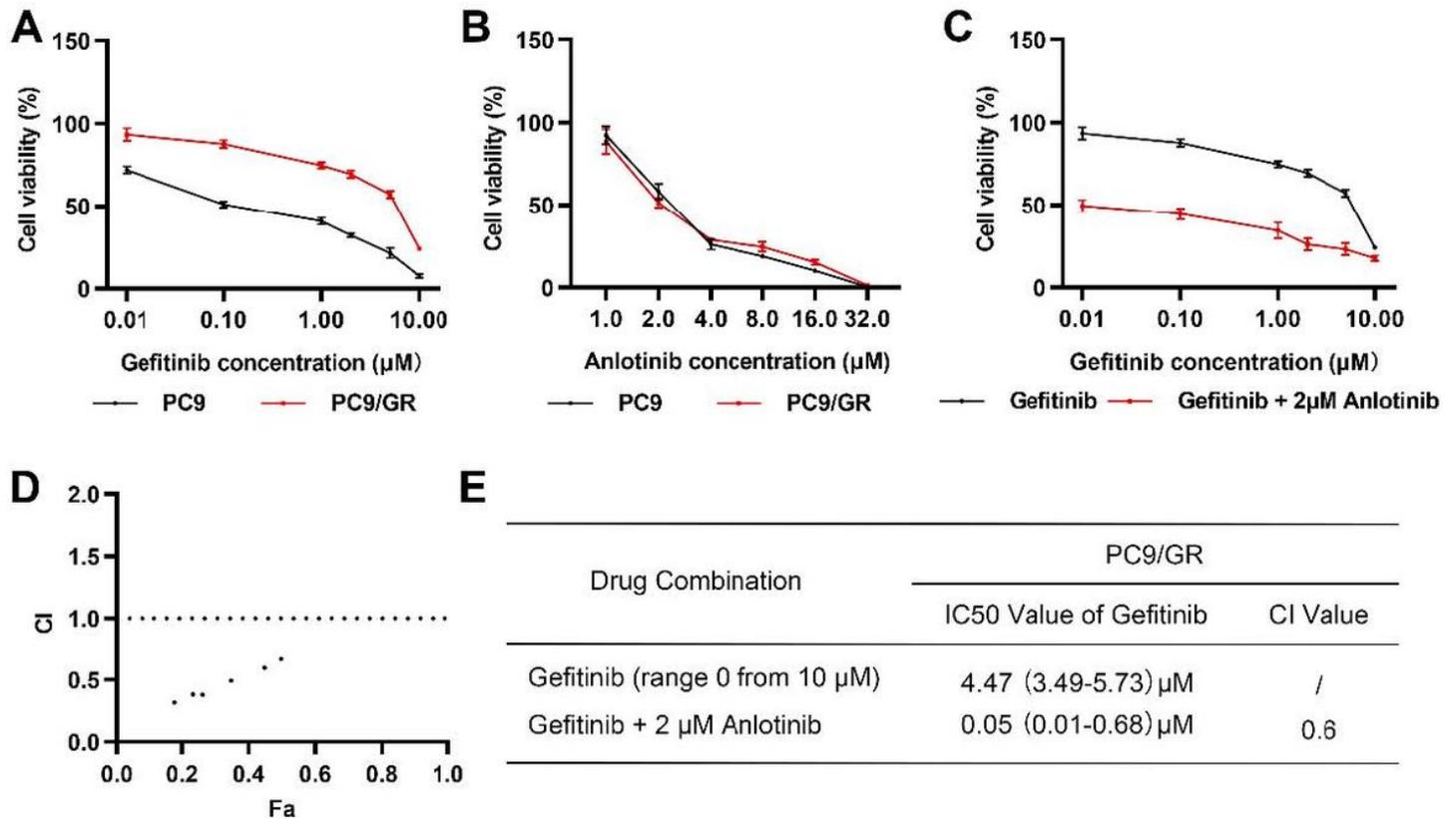


Figure 1

Anlotinib resensitized gefitinib-resistant PC9/GR cells to gefitinib. Parental lung adenocarcinoma PC9 cells and gefitinib-resistant PC9/GR cells were treated with indicated concentrations of gefitinib (A), anlotinib (B), or gefitinib plus anlotinib (C) for 72 h. Cell viability was measured by the CCK8 assay. D, The combination index of gefitinib plus anlotinib was calculated using CompuSyn software. E, Half maximal inhibitory concentration (IC₅₀) and combination index (CI) values of gefitinib alone or combined with anlotinib in PC9/GR cells.

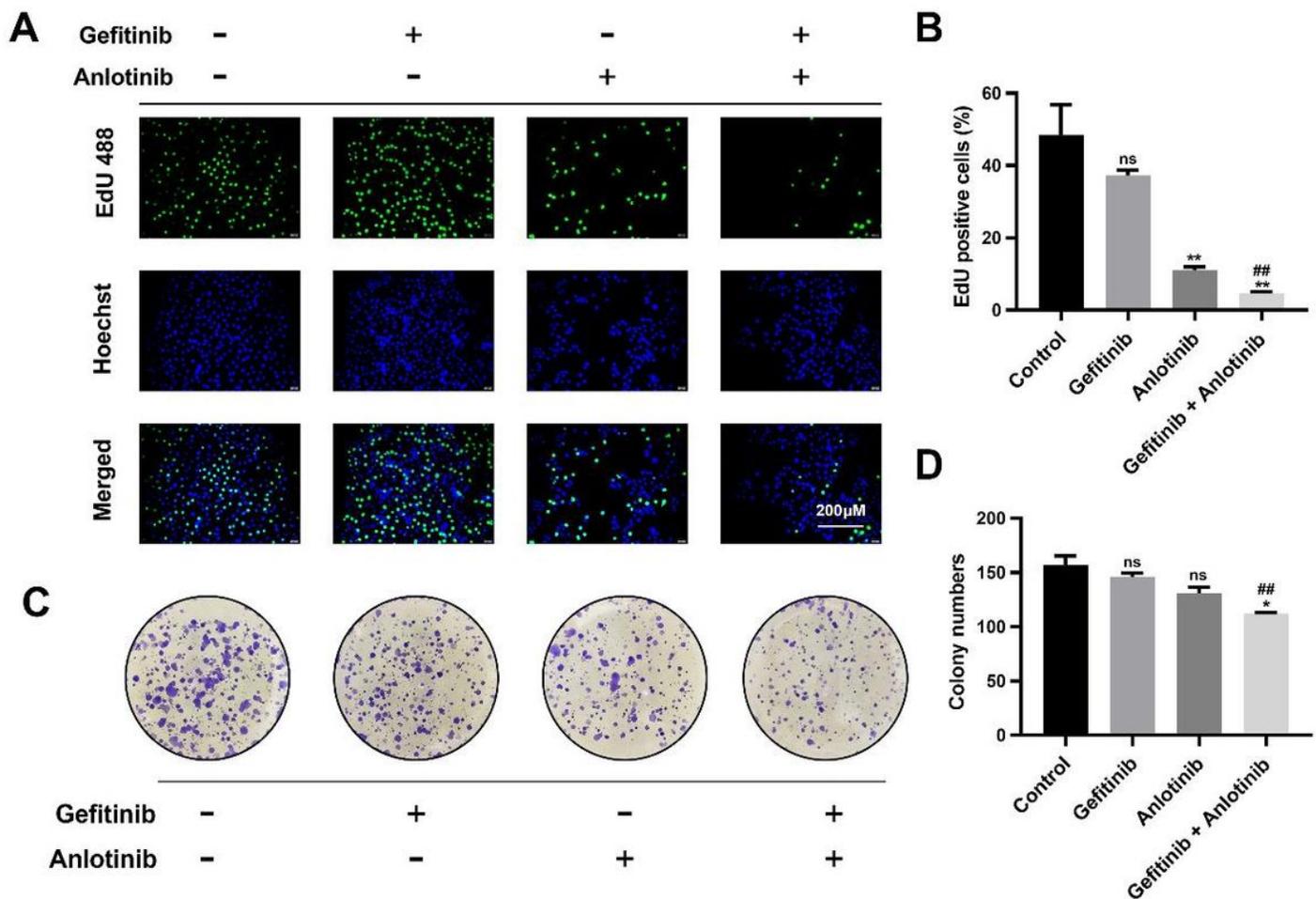


Figure 2

Treatment with gefitinib plus anlotinib synergistically inhibited proliferation of PC9/GR cells. A and B, EdU proliferation assays were performed 48 h after treatment with gefitinib, anlotinib, and gefitinib plus anlotinib. C and D, Colony-formation assays were performed to analyze the colony-formation efficiency of PC9/GR cells in the different treatment groups. Results exhibited as mean \pm SD of three independent experiments performed in triplicates. Significance levels determined using the t test are indicated (ns: not significant compared with the control group. * $P < 0.01$, ** $P < 0.001$ compared with the control group. # $P < 0.01$, ## $P < 0.001$ compared with the gefitinib group).

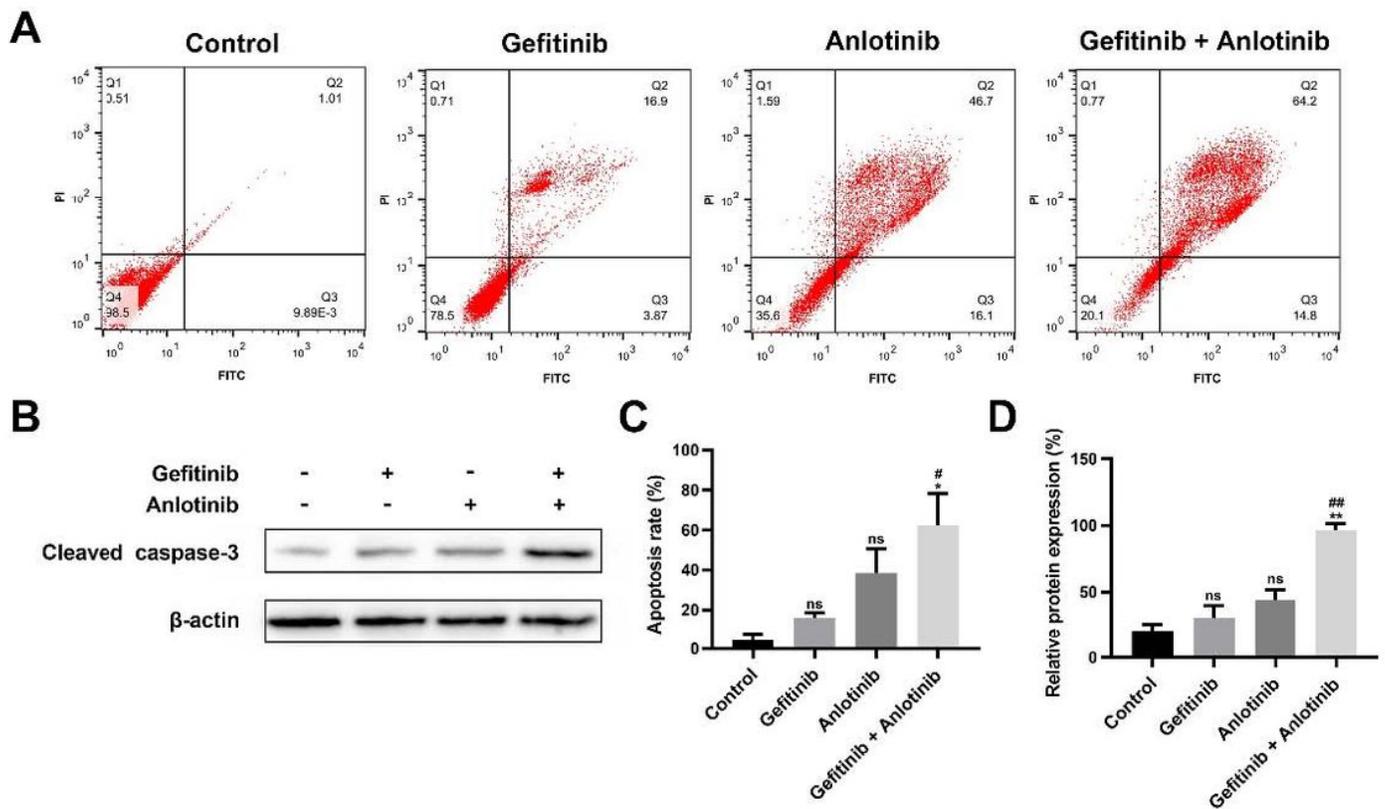


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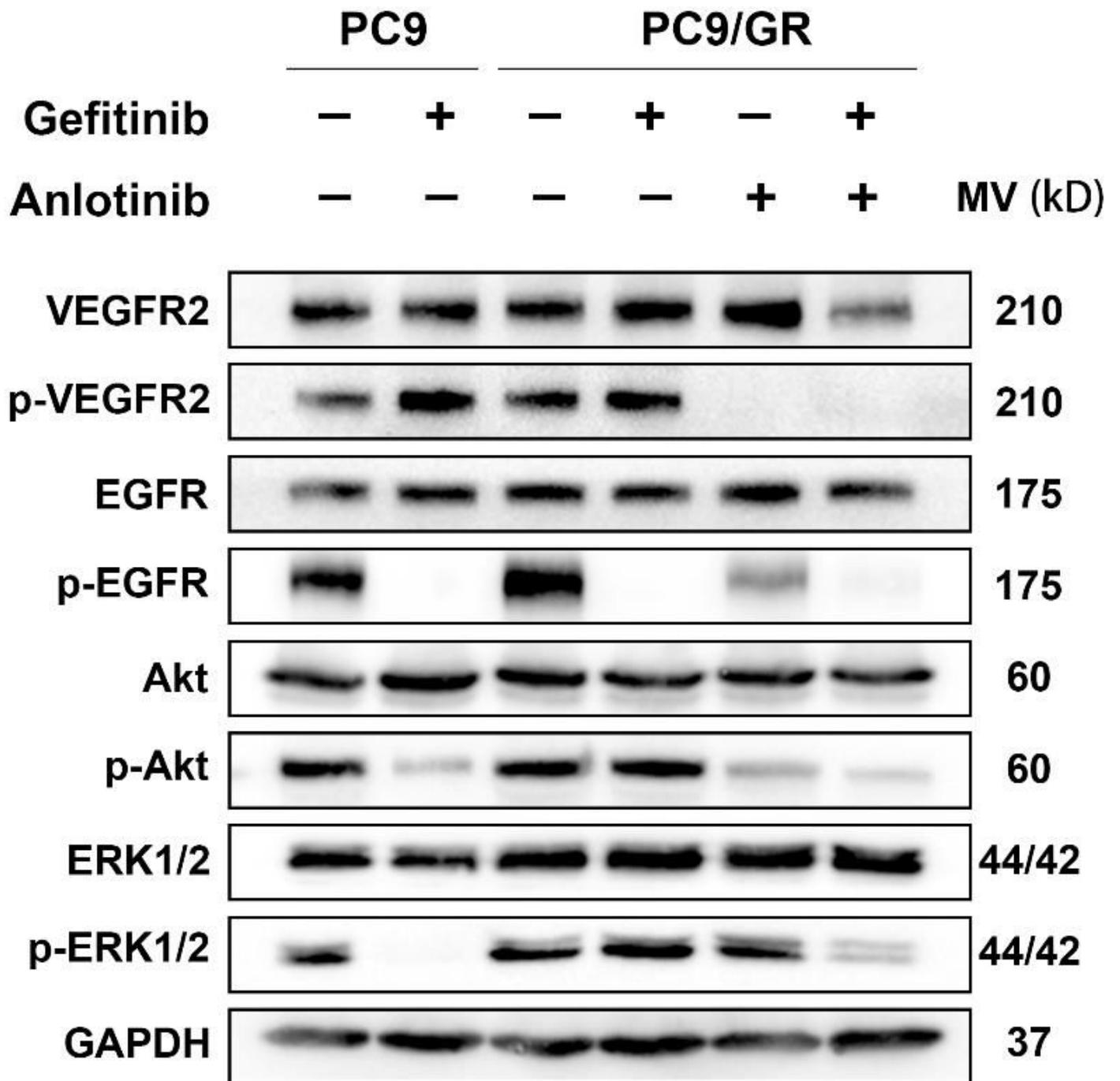


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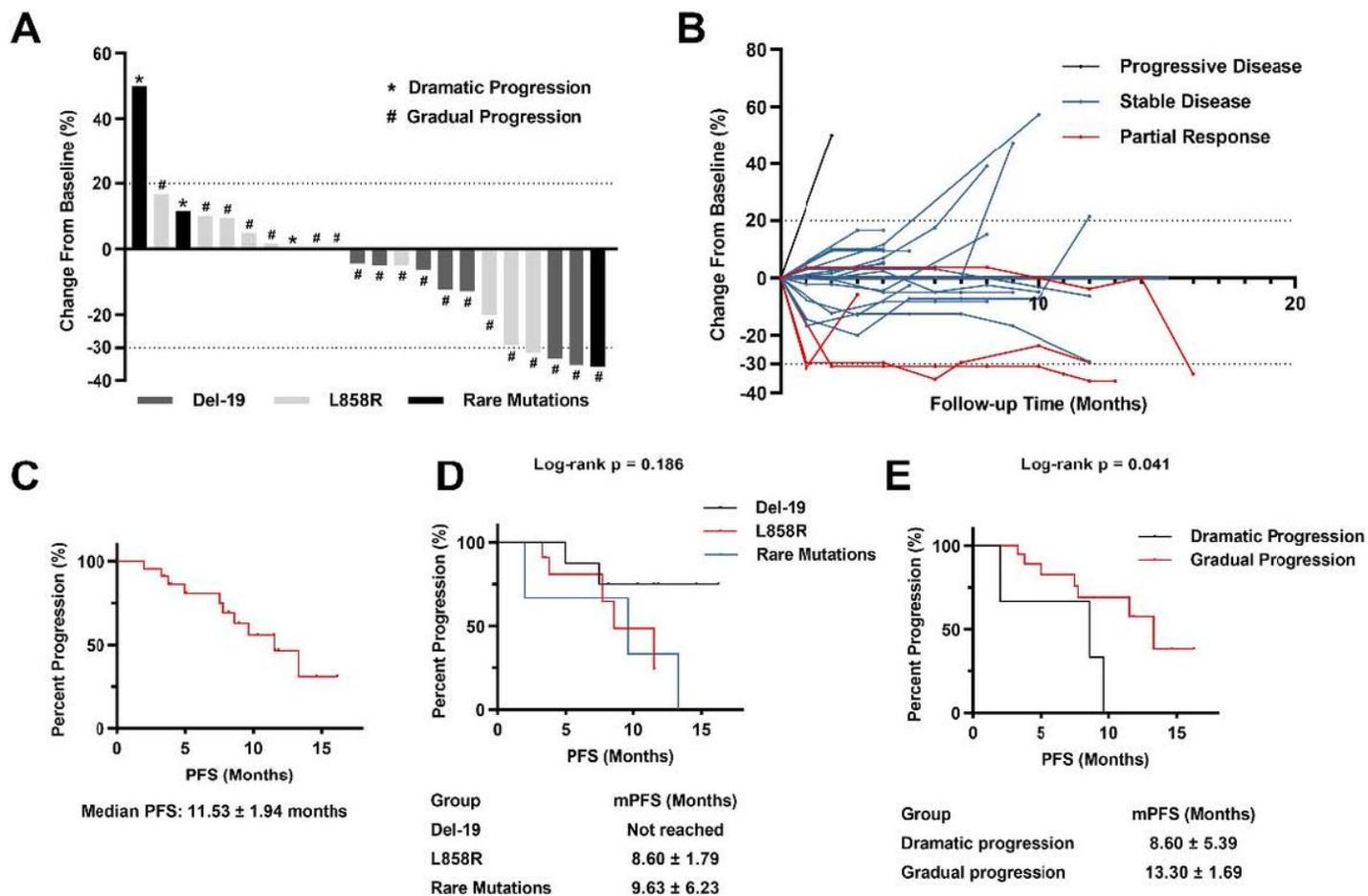


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