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RESEARCH

RAF1 promotes anlotinib resistance in non-small cell lung cancer by inhibiting apoptosis

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Abstract

Background Anlotinib is an effective treatment for advanced non-small cell lung cancer (NSCLC), but resistance to it often develops during therapy. RAF1, a serine/threonine kinase involved in cancer progression, has limited research in NSCLC, particularly regarding anlotinib resistance.

Methods Analysis of RAF1 expression in NSCLC and its relationship with targeted therapy resistance and apoptosis through bioinformatics methods. Immunohistochemistry (IHC) was employed to evaluate the relationship between RAF1 expression and anlotinib resistance in NSCLC tissues. Anlotinib-resistant PC9 (PC9/AR) cells were constructed in vitro, and cell viability and apoptosis were assessed using the cell counting kit-8 (CCK-8) assay and flow cytometry. Quantitative real-time PCR (qRT-PCR) was carried out to evaluate RAF1 gene expression levels, and western blot (WB) analysis was conducted to determine the expression of RAF1, Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2).

Results Bioinformatics analysis showed that RAF1 was lowly expressed in lung cancer tissues in TCGA and GEPIA databases. Further pathway analysis indicated that RAF1 expression was positively correlated with targeted therapy resistance and negatively correlated with the expression of the anti-apoptotic protein Bcl-2. Immunohistochemical analysis showed that high RAF1 expression in NSCLC tissues was related to anlotinib resistance ($P < 0.05$). In vitro experiments demonstrated that RAF1 contributed to anlotinib resistance in NSCLC cells. Overexpression of RAF1 increased cell viability and decreased apoptosis in PC9 and PC9/AR cells, while knockdown of RAF1 had the opposite effects.

Conclusion RAF1 mediates anlotinib resistance in NSCLC cells by regulating apoptosis and may serve as a predictive marker for anlotinib resistance in advanced lung cancer patients.

Keywords RAF1 · Anlotinib · NSCLC · Resistance · Apoptosis

Introduction

According to cancer statistics from 2025, lung cancer remains the leading cause of cancer-related deaths and is projected to have the highest mortality rate among all cancers (Siegel et al. 2025). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers, with approximately 70% of patients diagnosed at an advanced stage, resulting in poor five-year survival rates (Travis et al. 2013). Besides traditional chemotherapy and radiotherapy, the discovery of oncogenic driver mutations and the introduction of immune checkpoint inhibitors have greatly changed the treatment landscape for advanced lung cancer, resulting in significant improvements in clinical outcomes (Hendriks et al. 2024). Nevertheless, the inevitable development of resistance still limits the prognosis of NSCLC patients (Miao et al. 2024).

Anlotinib, an oral multi-target receptor tyrosine kinase inhibitor, inhibits angiogenesis and signaling pathways involved in cell proliferation to suppress tumor growth (Lu et al. 2019). It was approved by the Chinese National Medical Products Administration (NMPA) in 2018 as a third-line therapy for NSCLC, providing a new treatment option for advanced lung cancer patients and extending their survival times (Syed 2018). As an innovative

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anticancer agent, anlotinib shows promising potential in cancer therapy, especially in combination regimens, due to its unique pharmacological mechanisms and favorable clinical efficacy. For instance, clinical evidence has shown that the combination of anlotinib with osimertinib exhibits significant efficacy in treating advanced NSCLC (Lei et al. 2023). Similarly, in NSCLC patients with epidermal growth factor receptor (EGFR) mutations who fail tyrosine kinase inhibitor (TKI) therapy, the combination of anlotinib and anti-PD-1/PD-L1 therapies has demonstrated good efficacy and tolerability. With ongoing research and accumulating clinical trial data, the indications for anlotinib are expected to expand further (Shi et al. 2024). Despite its success, resistance to anlotinib has emerged as a significant limitation, and the underlying mechanisms remain incompletely understood. Although some studies have explored the mechanisms of anlotinib resistance, further research is needed to fully elucidate these mechanisms and develop predictive biomarkers to optimize clinical use (Chen et al. 2024).

Our research group has previously focused on identifying molecular biomarkers for monitoring the efficacy of anlotinib, aiming to discover precise and effective indicators for therapeutic response and to explore their potential mechanisms of action (Gu et al. 2021; Liu et al. 2022). Preliminary studies have suggested that the expression of RAF1 protein may be associated with anlotinib resistance. RAF1, a member of the serine/threonine-specific protein kinase family, is part of the RAS-activated enzymes that initiate signal transduction via the mitogen-activated protein kinase (MAPK) cascade, controlling cell proliferation, differentiation, and survival (Lim et al. 2023). RAF1 has been characterized as an oncogene, with its role in cancer not limited to the classical MAPK signaling pathway but also involving diverse kinase-dependent and kinase-independent functions (Drosten and Barbacid 2020). For example, RAF1 can regulate apoptosis through non-MAPK-dependent mechanisms, including direct binding and inhibition of pro-apoptotic proteins, apoptosis signal-regulating kinase 1 (ASK-1) and mammalian sterile 20-like kinase 2 (MST-2). Additionally, RAF1 knockdown has been shown to suppress Bcl-2 expression and induce apoptosis in melanoma cells (Riaud et al. 2024; O'Neill et al. 2004; Chen et al. 2001).

Apoptosis is closely associated with resistance to cancer therapies. The efficacy of cancer treatment depends not only on the damage induced by the therapy but also on the ability of cells to activate apoptotic programs (Kalkavan et al. 2022). While normal cells regulate apoptosis through intrinsic and extrinsic pathways, tumor cells evade apoptosis to develop resistance to anticancer therapies (Tian et al. 2024). However, the expression and role of RAF1 in NSCLC patients with resistance to anlotinib treatment remain unclear. This study investigates the differential expression of RAF1 in anlotinib-resistant NSCLC and examines whether RAF1 promotes resistance via the apoptotic pathway.

Materials and methods

Data source and preprocessing

Transcriptomic data of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) were obtained from The Cancer Genome Atlas (TCGA) database, including expression profiles of tumor and normal tissues. Transcripts per million (TPM) expression values were extracted and log2(TPM + 1) transformed for subsequent analyses.

Bioinformatics analysis

1. Differential gene expression analysis
   The expression levels of RAF1 in LUAD and LUSC tissues were analyzed using the TCGA database (https://portal.gdc.cancer.gov/) to compare tumor and normal tissues. Additionally, the expression results of RAF1 were validated using the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/). GEPIA analysis parameters were set as follows: |Log2FC| cutoff = 0.6 and P-value cutoff = 0.01.
2. Gene set enrichment analysis (GSEA)
   Single-sample gene set enrichment analysis (ssGSEA) was performed using the GSVA package in R software (version 4.4.2).
3. Apoptotic pathway analysis
   STAR-counts data and clinical information for LUAD and LUSC samples were downloaded and processed from the TCGA database. Apoptotic pathway activity was assessed using the ssGSEA method.

Patients

This study included 38 patients with advanced NSCLC who underwent lung biopsy or surgery at Lianyungang Clinical Medical College of Nanjing Medical University and were treated with oral anlotinib capsules between January 2020 and February 2024. Immunohistochemistry (IHC) was performed on tissue samples after morphologic evaluation of hematoxylin and eosin (H&E)-stained sections. Written informed consent was obtained from all patients prior to tissue collection, and the study was approved by the Ethics Committee of Lianyungang First People's Hospital (Approval No. KY-20241018003-01).

Immunohistochemistry

Paraffin-embedded tissue sections were baked, deparaffinized, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was blocked using a peroxidase blocking reagent, followed by blocking with goat serum. The sections were incubated overnight at 4 °C with primary antibodies (Wuhan Sanying, China). The next day, biotin-labeled secondary antibodies (Dako, Denmark) were applied, and sections were visualized with 3,3′-diaminobenzidine (DAB) for 3 min. Nuclei were counterstained with hematoxylin for 3 min, dehydrated with graded ethanol, cleared with xylene, and mounted with neutral resin. IHC images were independently evaluated by two experienced pathologists. Staining scores were determined based on: (1) percentage of positive cells: 0 (< 1%), 1 (1%–10%), 2 (11%–50%), 3 (51%–100%), and (2) staining intensity: 0 (negative), 1 (light yellow), 2 (brownish-yellow), 3 (dark brown). Final scores were calculated by multiplying the two scores, yielding values of 0, 1, 2, 3, 6, or 9. Samples were categorized as negative (0), low expression (1–2), moderate expression (3–6), or high expression (9).

Construction of anlotinib-resistant PC9 cells

PC9 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). To establish PC9/AR cells, PC9 cells were treated intermittently with gradient concentrations of anlotinib for over two months, with medium refreshed daily. Viable cells were harvested and used for functional assays.

Cell culture

PC9 and PC9/AR cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin solution. Cells were maintained in a humidified incubator at 37 °C with 5% CO2.

Cell proliferation and viability analysis

Cell counting kit-8 assay

Cell proliferation was measured using the CCK-8 assay kit (C6005, NCM Biotech). To evaluate the effect of anlotinib on cell growth, PC9 cells (5×10^4 cells/well) were seeded in 96-well plates and treated with anlotinib (0, 1, 2, 5, 10, or 20 μM) for 72 h. PC9/AR cells were treated with anlotinib (0, 20, 40, 80, 160, or 320 μM) for 72 h. Subsequently, 10 μl of CCK-8 solution was added and incubated for 2 h. Absorbance at 450 nm was measured using a microplate reader (DR-200Bs, Diatek).

Flow cytometry

PC9 and PC9/AR cells were digested with trypsin (C100C1, NCM Biotech), washed with PBS, and centrifuged at 1200 rpm for 5 min. Apoptotic cells were detected using the Annexin V-FITC apoptosis detection kit (C1062M, Beyotime) and analyzed with a BD Biosciences FACS Calibur system (San Jose, CA).

Cell transfection

PC9 and PC9/AR cells were transfected with RAF1 plasmids or RAF1-siRNA and their respective controls (control plasmids and control-siRNA) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. All plasmids were synthesized by GenePharma (Shanghai, China).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from PC9 and PC9/AR cells using TRIpure total RNA extraction reagent (YFXM0011P, Yifeixue Biotechnology) according to the manufacturer's instructions. HiScript III RT SuperMix (+gDNA wiper) (R323, Vazyme) was used for reverse transcription. Real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme) on a CG Real-Time PCR system (Heal Force). GAPDH served as the reference gene. Relative RAF1 expression was analyzed using the $2^{-\Delta\Delta CT}$ method.

Primer sequences for qRT-PCR:

Gene	Forward primer	Reverse primer
HomoRAF1	TGTCCAGTAGCC CCAACAAT	TCTCCGTGCCAT TTACCCTT
HomoGAPDH	AGGTCGGAGTCA ACGGATTT	TGACGGTGCCAT GGAATTTG

Western blotting

Total protein was extracted using RIPA lysis buffer (89901, Thermo Fisher Scientific). Protein concentrations were quantified using a BCA protein assay kit (P0010, Beyotime Biotechnology). Lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes (IPVH00010, Millipore). Membranes were blocked with skim milk and incubated overnight at 4 °C with specific primary antibodies, including anti-RAF1 (26863-1-AP, Proteintech, 1:1000), anti-Bax (50599-2-Ig, Proteintech, 1:2000), anti-Bcl-2 (26593-1-AP,

Proteintech, 1:1000), and anti-GAPDH (60004-1-Ig, Proteintech, 1:5000). Subsequently, membranes were incubated with secondary antibodies (7076, CST, 1:1000) and visualized using ECL reagents (P10010, NCM Biotech).

Statistical analysis

All bioinformatics analyses were conducted using R software (version 4.0.3), and correlations were analyzed using Spearman correlation analysis. Statistical analyses were performed using SPSS version 27.0 and GraphPad Prism version 8.0 software. Fisher's exact test was used to analyze the association between RAF1 expression and anlotinib efficacy. Cell experimental data were presented as mean ± standard deviation (SD). Differences were calculated using Student's t-test or one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

Results

RAF1 is associated with targeted therapy resistance and apoptosis in NSCLC

Analysis of transcriptomic data from TCGA revealed that RAF1 expression was significantly downregulated in tumor tissues compared to normal tissues in both LUAD and LUSC (Fig. 1A1, A2). This finding was further validated using the GEPIA database (Fig. 1A3). To investigate the potential relationship between RAF1 and NSCLC resistance, ssGSEA was performed to assess the correlation between RAF1 expression and pathways related to EGFR TKI resistance and apoptosis. Results indicated a positive correlation between RAF1 expression and the TKI resistance pathway ($R = 0.42$, $P < 0.05$), suggesting that RAF1 may contribute to NSCLC resistance to targeted therapy (Fig. 1B1, B2). Furthermore, RAF1 expression was negatively correlated with apoptosis and significantly associated with the expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) ($R = 0.48$, $P < 0.05$), while no significant correlation was observed with pro-apoptotic protein Bcl-2-associated X protein (Bax) (Fig. 1C1, C3). These findings suggest that RAF1 may suppress apoptosis by regulating Bcl-2 expression. In summary, RAF1 is downregulated in NSCLC and closely associated with both the EGFR and apoptosis pathways, potentially playing a critical role in anlotinib resistance.

Immunohistochemical validation of the correlation between RAF1 expression and anlotinib resistance

This study included 38 patients, and representative IHC staining images showing no expression, high expression, moderate expression, and low expression of RAF1 in lung cancer tissues are presented in Fig. 2. Among these, 14 patients (36.8%) exhibited high RAF1 expression, 8 (21.1%) had moderate expression, 9 (23.7%) had low expression, and 7 (18.4%) had no expression. Demographic and clinical characteristics of the 31 RAF1-positive patients are summarized in Table 1, with a median age of 71 years. Among these, 19 patients (61.3%) exhibited anlotinib resistance, while 12 patients (38.7%) were sensitive to the treatment. At the end of the observation period, the number of anlotinib-resistant patients in the high, moderate, and low RAF1 expression groups was 12 (85.7%), 4 (50%), and 3 (33.3%), respectively (Fig. 2). These differences were statistically significant ($\chi^2 = 6.870$, $P = 0.037$), indicating that increased RAF1 expression in tumor cells is associated with anlotinib resistance.

RAF1 is involved in anlotinib resistance in PC9 and PC9/AR cells

To confirm the successful establishment of the resistant cell line, we evaluated the viability of PC9 and PC9/AR cells after 72 h of treatment with varying concentrations of anlotinib. Anlotinib decreased cell viability in a dose-dependent manner in both PC9 and PC9/AR cells (Figs. 3A, B). The IC50 value of anlotinib in PC9 cells was 9.49 μM, while in PC9/AR cells, it increased to 39.57 μM (Fig. 3C). Accordingly, the resistance index (RI) of PC9/AR cells was calculated as 4.17, indicating a significantly higher resistance to anlotinib compared to PC9 cells. These results confirm the successful establishment of the resistant cell line.

Next, we examined whether RAF1 plays a regulatory role in anlotinib resistance. Results from qRT-PCR and WB analyses showed higher RAF1 levels in PC9/AR cells compared to PC9 cells (Figs. 3D–F). Additionally, anlotinib significantly inhibited RAF1 mRNA levels in a dose-dependent manner in both PC9 (Fig. 3G) and PC9/AR cells (Fig. 3J). WB revealed similar trends in protein expression in PC9 (Figs. 3H, I) and PC9/AR cells (Figs. 3K, L). Collectively, these findings indicate that RAF1 contributes to anlotinib resistance in PC9/AR cells.

RAF1 regulates cell viability and apoptosis in NSCLC cells

To explore the potential functions of RAF1 in NSCLC cells, we overexpressed RAF1 using pcDNA3.1-RAF1 and knocked down RAF1 using RAF1-siRNA, with pc-NC and control-siRNA as scrambled controls. Transfection efficiency was confirmed via qRT-PCR and WB analyses (Supplementary Fig. 1), showing increased RAF1 mRNA and protein levels in RAF1-overexpressing cells and significantly decreased RAF1 levels in RAF1-knockdown cells.

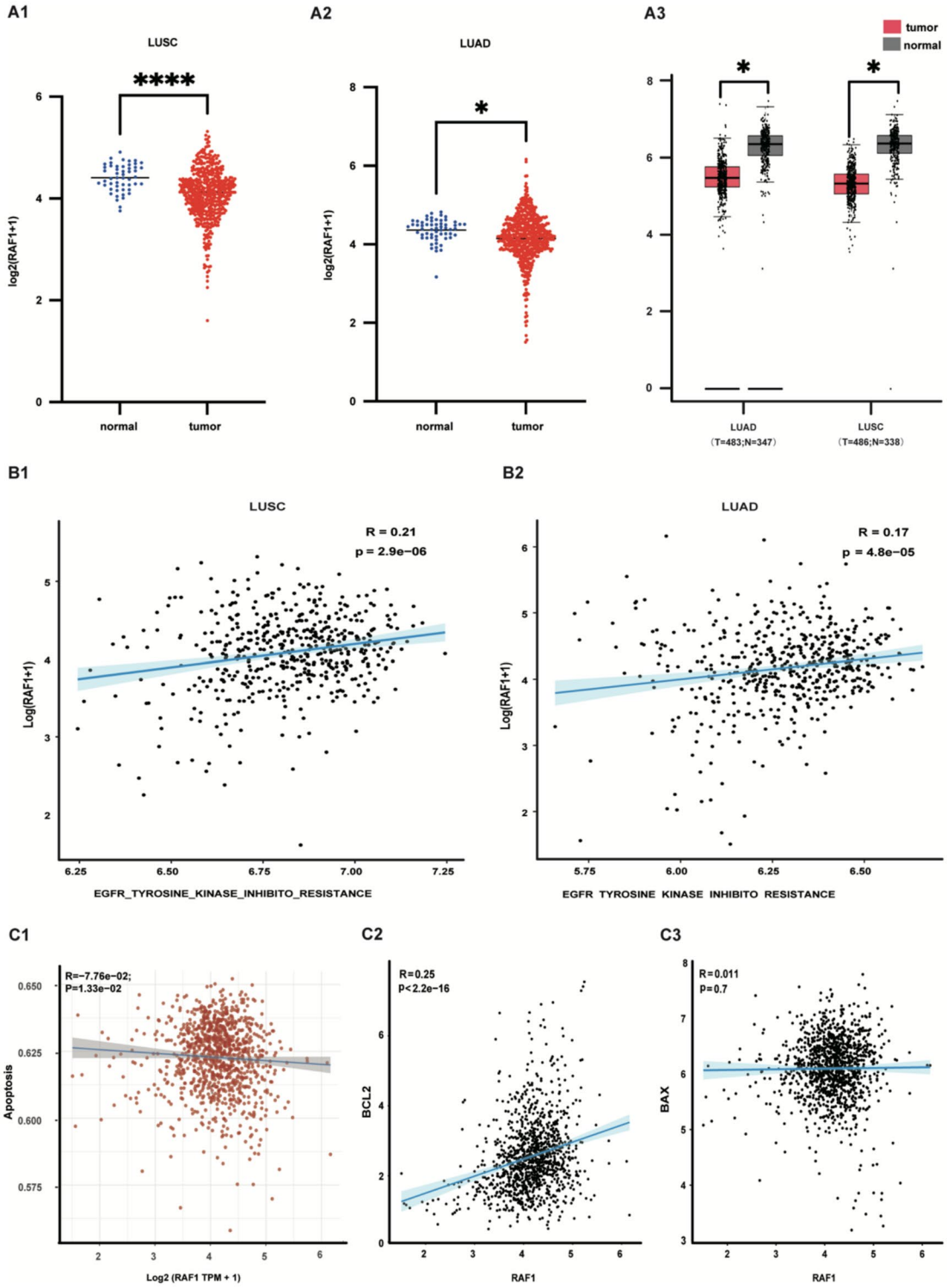

Fig. 1 Correlation of RAF1 with resistance to targeted therapy for NSCLC and apoptosis. **A1**, **A2**, **A3** Expression levels of RAF1 in LUAD and LUSC tissues. **B1**, **B2** In LUAD and LUSC, RAF1 expression is positively correlated with the TKI resistance pathway. **C1** Spearman correlation analysis shows a negative correlation between RAF1 expression and cell apoptosis (the x-axis represents the distribution of the expression of gene RAF1, and the y-axis represents the distribution of the pathway score). **C2**, **C3** Anti-apoptotic protein Bcl2 expression is positively correlated with RAF1, while pro-apoptotic protein Bax shows no significant correlation with RAF1. *T* tumor tissue sample, *N* normal tissue sample. *P < 0.05, ****P < 0.0001. *LUAD* lung adenocarcinoma, *LUSC* lung squamous cell carcinoma

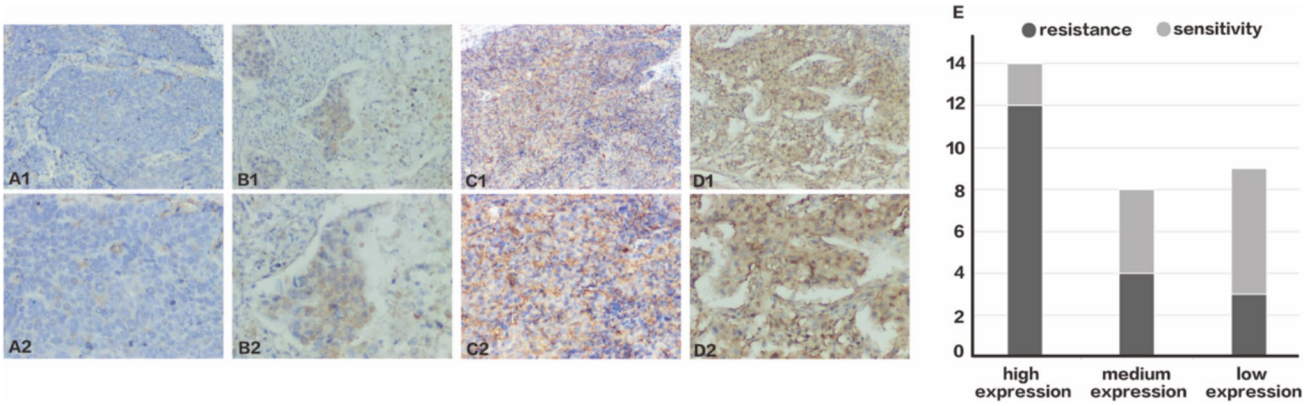

Fig. 2 Correlation between RAF1 expression levels and anlotinib resistance. **A–D** Immunohistochemical staining for RAF1. From **A–D** negative (−), low expression (+), medium expression (++), and high expression (+++). A1-D1 × 100, A2-D2 × 200. (E)High RAF1 expression group (n = 14, resistant = 12); medium RAF1 expression group (n = 8, resistant = 4); low RAF1 expression group (n = 9, resistant = 3). Data are represented as the number of cases within each group categorized by resistance status

Table 1 Clinical and pathological characteristics of the patients

Factor	N (%) Total n = 31
Sex	
Male	23 (74.2%)
Female	8 (25.8%)
Age, median (range, years)	71(33–86)
Histological subtype	
Adenocarcinoma	20(64.5%)
Squamous	11(35.5%)
Tumor size	
≤ T2	20(64.5%)
> T2	11(35.5%)
Combination medications	
Yes	16(51.6%)
No	15(48.4%)
Mutation	
Yes	11(35.5%)
No	20(64.5%)
Anlotinib resistance	
Yes	19(61.3%)
No	12(38.7%)

CCK-8 assays revealed that RAF1 overexpression significantly enhanced cell viability in both PC9 and PC9/AR cells compared to control/pc-NC-treated cells (Fig. 4A1), while RAF1 knockdown exhibited the opposite effect (Fig. 4A2). Flow cytometry showed decreased apoptosis in RAF1-overexpressing cells (Figs. 4B1, C1) and increased apoptosis in RAF1-knockdown cells (Figs. 4B2, C2). WB analysis demonstrated that RAF1 overexpression reduced Bax expression and significantly increased Bcl-2 expression in PC9 and PC9/AR cells (Figs. 4D1, E1). Conversely, RAF1 knockdown elevated Bax expression and suppressed Bcl-2 expression (Figs. 4D2, E2). These observations suggest that RAF1 regulates cell viability and apoptosis in PC9 and PC9/AR cells.

RAF1 overexpression inhibits anlotinib resistance by modulating cell proliferation and apoptosis

To investigate whether RAF1 modulates anlotinib resistance in NSCLC cells through the apoptosis pathway, rescue experiments were performed. PC9 and PC9/AR cells treated with anlotinib, which suppressed RAF1 mRNA and protein expression, were transfected with RAF1-plasmid or control-plasmid. qRT-PCR and WB analyses confirmed that RAF1 overexpression significantly restored RAF1 mRNA and protein levels in anlotinib-treated cells (Supplementary Fig. 2). Subsequent assays demonstrated that RAF1 overexpression reversed anlotinib-induced apoptosis and restored cell viability (Figs. 5A–C). Further WB analysis showed that anlotinib significantly increased Bax expression and reduced Bcl-2 expression, effects that were partially rescued by RAF1 overexpression (Figs. 5D, E). Overall, our results indicate that RAF1 overexpression inhibits anlotinib resistance in NSCLC by regulating cell proliferation and apoptosis.

Discussion

Anlotinib, a small-molecule multi-target tyrosine kinase inhibitor, is currently approved as a third-line treatment for patients with advanced NSCLC (Syed 2018). While anlotinib has improved progression-free survival (PFS) and overall survival (OS), resistance to the drug is inevitable (Han et al. 2018). Identifying potential mechanisms of resistance and predictive biomarkers has thus become a critical

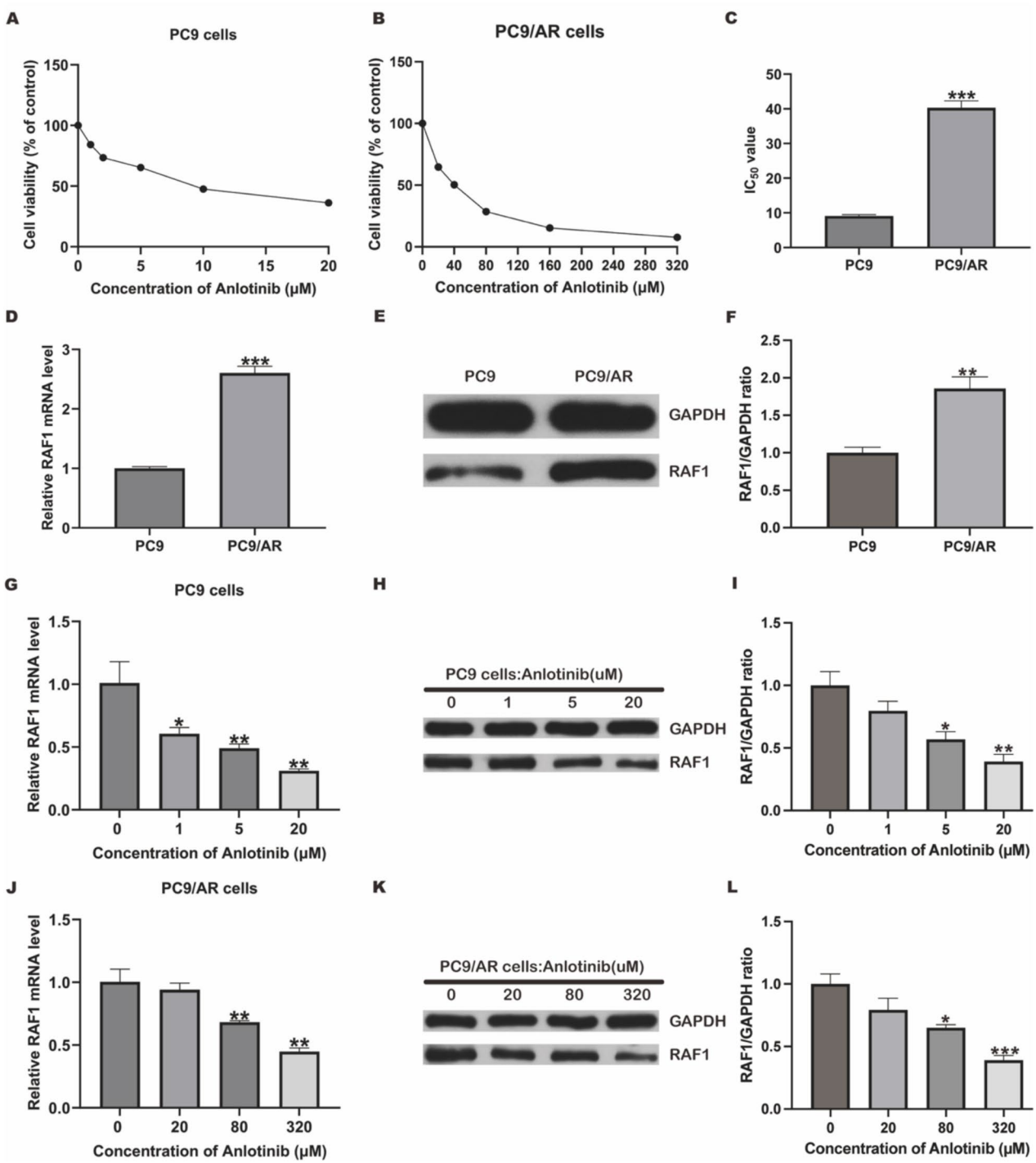

Fig. 3 Effects of anlotinib on the expression of RAF1 expression in PC9 and PC9/AR cells. PC9 and PC9/AR cells were exposed to different concentrations of anlotinib. **A** The viability of PC9 cells under different concentrations of anlotinib (0, 1, 2, 5, 10 or 20 μM) was determined using CCK-8 assay. **B** The proliferation of PC9/AR cells were treated with anlotinib (0, 20, 40, 80, 160 or 320 μM) was measured using CCK8 assay. **C** The IC50 value of PC9 and PC9/AR cells were assessed. **D** RAF1 mRNA level was detected by qRT-PCR. **E** WB was used to detect RAF1 protein expression. **F** Quantification of RAF1/GAPDH ratio. The relative mRNA level was determined using qRT-PCR in anlotinib treated PC9 cells (**G**) and PC9/AR cells (**J**). The RAF1 protein expression in anlotinib treated PC9 cells (**H**) and PC9/AR cells (**K**) was assessed using WB. Quantification of RAF1/GAPDH ratio in PC9 cells (**I**) and PC9/AR cells (**L**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

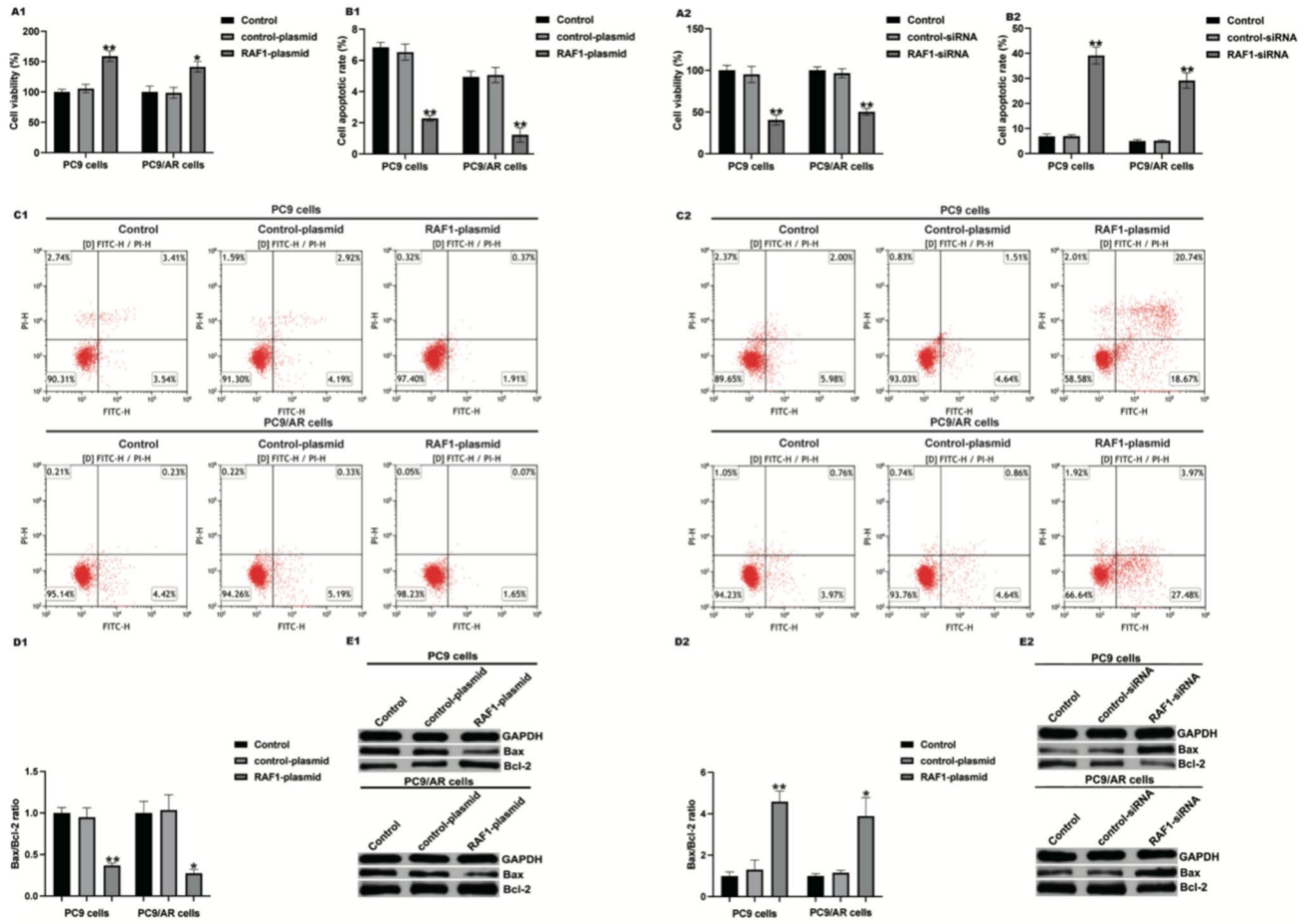

Fig. 4 Effects of RAF1 overexpression and knockdown on PC9 and PC9/AR cells viability and apoptosis. PC9 and PC9/AR cells were transfected with control plasmid or RAF1 plasmid/ control-siRNA or RAF1- siRNA. **A1**, **A2** Cell viabilities were evaluated using CCK-8. **B1**, **B2** Quantification of apoptotic cells. **C1**, **C2** Cells apoptosis were assessed by flow cytometry. **D1**, **D2** Quantification of Bax/Bcl-2 ratio. **E1**, **E2** Bax and Bcl-2 expression was detected by WB. *P < 0.05, **P < 0.01, ***P < 0.001

challenge. In our previous studies, we observed that RAF1 protein expression might be associated with anlotinib resistance. To validate this, we performed bioinformatic analyses and found that RAF1 expression in NSCLC tumor tissues is associated with targeted therapy resistance and apoptotic pathways. IHC further confirmed the clinical relevance of these differences. Moreover, experiments on PC9-resistant cells demonstrated that RAF1 promotes resistance to anlotinib in PC9 and PC9/AR cells by inhibiting apoptosis.

Several studies have investigated the mechanisms underlying anlotinib resistance and potential strategies to overcome it. For instance, SOD2 was shown to protect mitochondria by reducing ROS production, contributing to anlotinib resistance in oral squamous cell carcinoma cells (Li et al. 2024). Similarly, PTEN loss and MAPK pathway reactivation have been implicated in the development of resistance (Chen et al. 2022). Wang et al. reported that astragaloside IV could reverse anlotinib resistance in NSCLC cells by inhibiting the miR-181a-3p/UPR-ERAD axis (Wang et al. 2024). Likewise, eIF4E activation was shown to reverse anlotinib resistance in NSCLC when inhibited by cephalostatin or other MNK inhibitors (Zhang et al. 2023). Despite these advances, studies focusing on the mechanisms of anlotinib resistance in lung cancer remain limited.

Through bioinformatics analysis, we preliminarily identified a potential link between RAF1 and resistance to anlotinib. Both the TCGA and GEPIA databases showed that, compared to normal lung tissue, the mRNA levels of RAF1 were significantly downregulated in LUAD and LUSC tissues. However, subsequent immunohistochemical tests and cell experiments indicated that the increased levels of RAF1 protein in cancer tissues and cells were associated with increased resistance. Previous studies have also observed this discrepancy between mRNA expression and protein function, which may be caused by complex regulatory mechanisms, including post-transcriptional regulation,

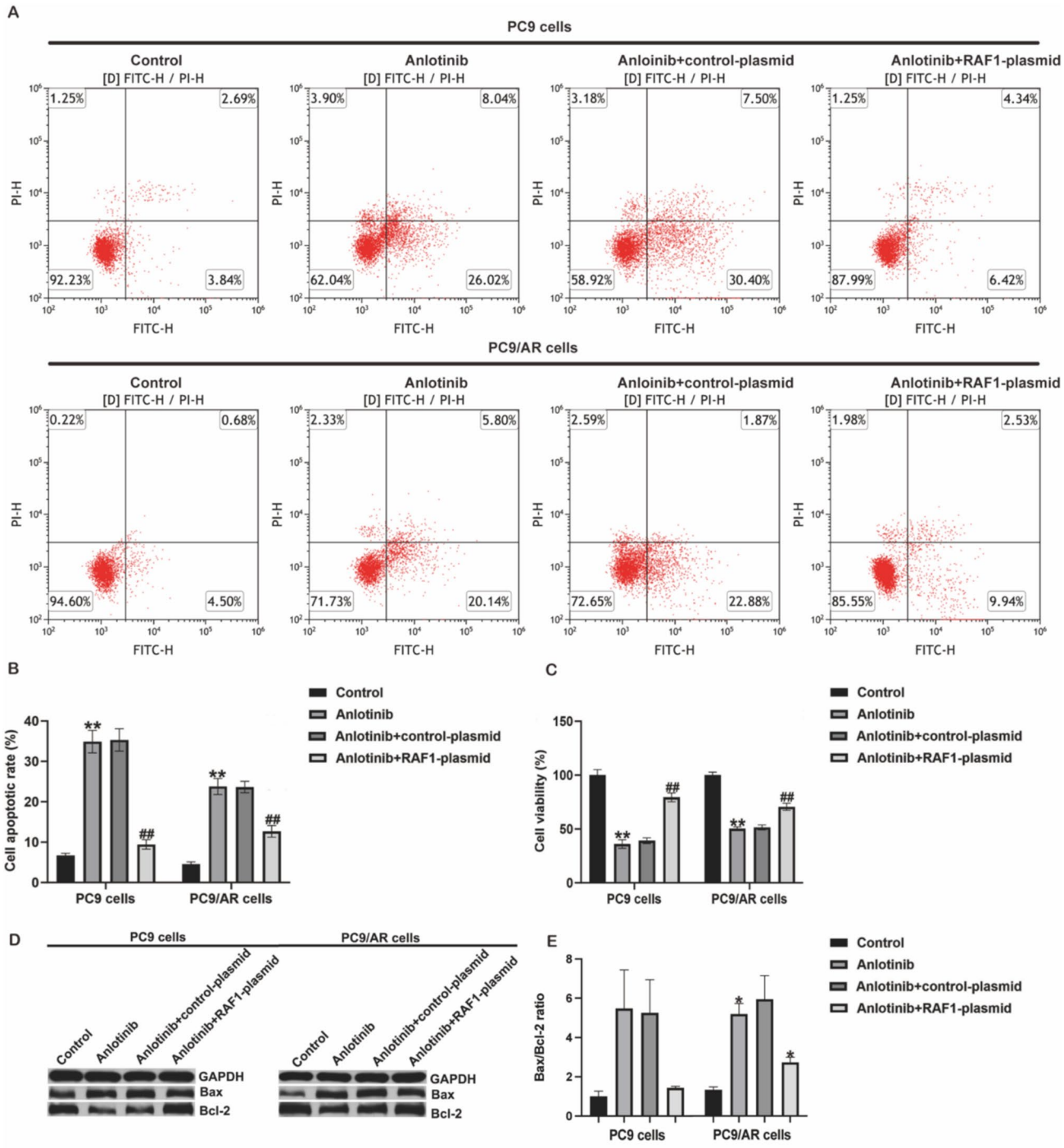

Fig. 5 Effects of RAF1 plasmid on anlotinib-induced PC9 and PC9/AR cells viability and apoptosis. PC9 and PC9/AR cells were transfected with control plasmid or RAF1 plasmid, followed by anlotinib treatment. **A**, **B** Cells apoptosis were assessed by flow cytometry. **C** CCK-8 assay was used to evaluate cells viability. **D** WB analysis of Bax and Bcl-2 protein expression. **E** Quantification of Bax/Bcl-2 ratio. *P < 0.05, **P < 0.01. ##P < 0.01

protein stability, and post-translational modifications (Desideri et al. 2015). It may also be due to tumor heterogeneity and the infiltration of non-cancer cells in a large number of tumor samples, which can obscure the true cancer-specific expression profile, making mRNA levels not always accurately reflect protein activity (Aran et al. 2015). In addition, our bioinformatics analysis indicated that RAF1 expression positively correlates with the TKI resistance pathway,

suggesting that RAF1 might modulate the response of NSCLC cells to targeted therapies via the EGFR signaling pathway. Additionally, Spearman correlation analysis revealed a significant positive correlation between RAF1 and the anti-apoptotic protein Bcl2, but no significant correlation with the pro-apoptotic protein Bax, consistent with previous findings (Jiang et al. 2024). However, the RAF1-apoptosis-anlotinib resistance axis in NSCLC has not been systematically studied. Based on these results, we hypothesize that RAF1 may promote cell survival by modulating apoptotic pathways, thereby playing a critical role in anlotinib resistance in NSCLC.

The RAF kinase family comprises three isoforms with distinct expression patterns, regulatory mechanisms, and roles in the Ras-MEK-ERK cascade. Among these, RAF1 is more ubiquitously expressed than ARAF and BRAF (Trakul and Rosner 2005). RAF1 is closely associated with the regulation of cell proliferation and differentiation and is abnormally activated in various tumors (Cobb et al. 1994). While BRAF is the most extensively studied RAF protein, the role of RAF1 in tumorigenesis and drug resistance has gained increasing clinical relevance (Riaud et al. 2024). For example, Tian et al. demonstrated a significant association between RAF1 expression and the invasive and metastatic potential of A549 and H1299 cell lines in lung cancer (Tian et al. 2018). To evaluate the clinical significance of RAF1 differential expression, our IHC analyses confirmed the correlation between RAF1 expression and resistance to anlotinib, with higher RAF1 expression associated with poorer therapeutic outcomes. For patients with advanced lung cancer, RAF1 IHC analysis before treatment might provide a predictive indicator of anlotinib efficacy. However, our study included a limited sample size and lacked stratified analysis of patients treated with monotherapy or combination therapy. Combination therapies can significantly extend patient survival. For example, Lei et al. demonstrated that in patients with advanced NSCLC resistant to osimertinib, anlotinib combined with other therapies could reverse resistance by targeting the c-MET/MYC/AXL axis, thereby improving survival (Lei et al. 2023). Stratified analysis of the efficacy of anlotinib in combination with chemotherapy, targeted therapy, or immunotherapy in advanced NSCLC may help identify optimal treatment strategies. Additionally, due to the invasive nature of biopsies, dynamic monitoring of therapeutic efficacy during treatment is challenging. Future studies could involve larger cohorts and utilize liquid biopsy techniques to monitor changes in RAF1 expression in circulating tumor cells in patients with anlotinib resistance or sensitivity. This approach could further elucidate the relationship between RAF1 and anlotinib resistance.

Both bioinformatics and IHC analyses indicate that RAF1 expression correlates with targeted therapy resistance. Subsequent cell-based experiments investigated how RAF1 influences anlotinib sensitivity in NSCLC. Using anlotinib-resistant cell lines treated with varying drug concentrations, we examined changes in RAF1 and apoptosis-related protein expression. We found that RAF1 overexpression suppressed the pro-apoptotic protein Bax and enhanced the anti-apoptotic protein Bcl-2, leading to increased resistance to anlotinib. Conversely, inhibiting RAF1 expression reversed resistance. These findings are consistent with our prior analyses. However, our experiments were limited to a single lung adenocarcinoma cell line (PC9) and did not include in vivo mouse models. Furthermore, the suppression of apoptosis by RAF1 overexpression may occur through the classical Ras-MEK-ERK cascade, although other pathways cannot be excluded. For example, Xu et al. showed that the Raf1-cdc25-cdc2 axis induces G2/M arrest and subsequent apoptosis in A549 cells (Xu et al. 2019). Similarly, Qiu et al. demonstrated that RAF1 regulates NSCLC tumor growth by inhibiting apoptosis via downstream effector p70S6K (Qiu et al. 2019). Further studies are required to elucidate the precise signaling pathways through which RAF1 mediates anlotinib resistance in NSCLC.

Conclusion

In this study, we demonstrated that RAF1 overexpression in NSCLC cells promotes resistance to anlotinib by inhibiting apoptosis. Additionally, we found that high RAF1 expression in lung cancer tissues may serve as a predictive marker for anlotinib resistance in lung cancer patients. These findings provide a novel perspective on the mechanisms underlying anlotinib resistance and suggest RAF1 as a potential target for monitoring resistance in patients with advanced NSCLC.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00432-025-06175-0.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SW, CXH, WH and PWH. The first draft of the manuscript was written by SW and all authors commented on previous versions of the manuscript. XDJ and KYH provided editorial input for the article. All authors read and approved the final manuscript.

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Availability of data and material No datasets were generated or analysed during the current study.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Collection and analysis of patient-level data for this article was approved by the Ethics Committee of Lianyungang First People's Hospital (Approval No. KY-20241018003–01).

Consent to participate The need for written consent from patients was waived per above approval protocols.

Consent for publication Not applicable.

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