

Combined treatment with N-acetylcysteine and gefitinib overcomes drug resistance to gefitinib in NSCLC cell line

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Abstract

We aimed to explore the molecular substrate underlying EGFR-TKI resistance and investigate the effects of N-acetylcysteine (NAC) on reversing EGFR-TKI resistance. In the current research, the effects of NAC in combination with gefitinib on reversing gefitinib resistance were examined using CCK-8 assay, combination index (CI) method, matrigel invasion assay, wound-healing assay, flow cytometry, western blot, and quantitative real-time PCR (qRT-PCR). CCK8 assay showed that NAC plus gefitinib combination overcame EGFR-TKI resistance in non-small cell lung cancer (NSCLC) cells by lowering the value of half maximal inhibitory concentration (IC50). CI calculations demonstrated a synergistic effect between the two drugs (CI < 1). Matrigel invasion assay and wound healing assay demonstrated a decrease in migration and invasion ability of PC-9/GR cells after NAC and gefitinib treatment. Flow cytometry displayed enhanced apoptosis in the combination group. Western blot and qRT-PCR revealed that increased E-cadherin and decreased vimentin in the combination group. When PP2 was administered with gefitinib, the same effects were seen. Our findings suggest that NAC could restore the sensitivity of gefitinib-resistant NSCLC cells to gefitinib via suppressing Src activation and reversing epithelial-mesenchymal transition.

KEYWORDS

epithelial-mesenchymal transition, gefitinib resistance, N-acetylcysteine, NSCLC, Src

1 | INTRODUCTION

Lung cancer, one of the most aggressive cancers, accounts for the leading cause of cancer-related mortality worldwide,^{1,2} and non-small cell lung cancer (NSCLC) contributes to around 85% of lung cancer. Gefitinib and erlotinib, two major epithelial growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), display promising therapeutic efficacy in patients with NSCLC carrying EGFR-activating mutations (for example, exon 19 deletion and exon 21 L858R), and

these patients regularly receive EGFR-TKIs as a first-line treatment.³⁻⁷ However, almost all patients become resistant to gefitinib and erlotinib within a median time period of approximately 10 months.⁸⁻¹⁰ However, the mechanisms of EGFR-TKI resistance are largely unknown. Therefore, exploring the underlying mechanisms is beneficial for developing new strategies to overcome this problem, which might improve the prognosis of patients with NSCLC.

Currently, multiple factors are involved in the resistance mechanisms, including the T790M secondary mutation in EGFR, HGF overexpression, MET amplification, transition into small cell lung carcinoma, obtaining cancer stem-cell

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phenotypes and epithelial-mesenchymal transition (EMT).¹¹⁻¹⁴ EMT is a universal phenomenon in various physiological and pathological processes. It is clear that epithelial cells display characteristics of mesenchymal cells, accompanied by upregulation of vimentin and N-cadherin, and downregulation of E-cadherin during EMT. EMT also contributes to tumor invasion, proliferation, metastasis, and therapy resistance to EGFR-TKIs.^{15,16} As a consequence, targeting EMT might be a potential strategy to reverse or prevent EGFR-TKIs resistance.

N-acetylcysteine (NAC) is an effective antioxidant widely used in anticancer investigation in recent years. Our previous studies have demonstrated that NAC could overcome gefitinib resistance mediated by cigarette smoke extract (CSE). However, whether NAC plays a critical role in non-smokers would explore the combined effect of NAC with gefitinib on gefitinib-resistant cells and the underlying mechanisms.

2 | MATERIALS AND METHODS

2.1 | Cell culture

PC-9 gefitinib-sensitive cells (PC-9)^{17,18} and gefitinib-resistant cells (PC-9/GR) were gifts from Dr Jian Zhang at Xijing Hospital, Fourth Military Medical University, China. It is known that exon19 deletion is one of the hall-marks in EGFR-activating mutation, and PC-9 cell line is characterized by exon19 deletion of lung cancer cells. The mutation profile is exon19(E746-A750)del for PC-9/GR.¹⁹ Cells were cultured in RPMI 1640 medium (Hyclone, USA) with 10% fetal bovine serum (PAN, USA) at 37°C in a cell incubator containing 5% CO₂. In addition, PC-9/GR cell was cultured in medium containing 10 nmol/L of gefitinib to maintain resistance.

2.2 | Reagents

NAC, 4',6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), and Triton X-100 were purchased from Sigma-Aldrich. PP2 and gefitinib were purchased from Tocris Bioscience and Abcam, respectively. Rabbit monoclonal antibodies against E-cadherin (24E10), Src (32G6), Phospho-Src (Ty416), and mouse monoclonal antibody against GAPDH were purchased from Cell Signaling Technology. Bax (WL01637) and Bcl-2 (WL01556) were purchased from Wanleibio. Rabbit monoclonal antibody against Vimentin (EGFR3776) was purchased from Abcam.

2.3 | Cell growth assay

Cell proliferation was evaluated with a CCK8 kit (Dojindo Laboratories). In brief, Cells (5×10^3 cells) were seeded into 96-well plates, cultured overnight and treated with various concentrations of drugs for 48 hours. Then, 10 μ L of CCK8

was added to each well and cells were incubated for 2 hours. Optical density (OD) was set at 450 nm by Microplate Reader.

2.4 | Combination studies

Combination studies were performed as described previously.^{20,21} On the basis of the median-effect analysis by Chou and Talalay (CalcuSyn software, Biosoft: Chou, 2010), the effects of drugs were calculated using the CI method for each experimental condition.^{22,23}

2.5 | Western blotting analysis

The precise procedure was in accordance with our previous methods.²⁴ In brief, proteins were separated by SDS-PAGE gel and transferred to PVDF membranes. After blocking with 5% fat-free milk or BSA, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 2 hours at room temperature. The protein bands were detected.

2.6 | RNA isolation and qRT-PCR

The procedure of RNA extraction and cDNA synthesis was based on our previous study.²⁵ The following primers were used: E-cadherin, 5-CGTAGCAGTGACGAATGTGG-3(F) and 5-CTGGGCAGTGTAGGATGTGA-3(R); vimentin, 5-GAGTCCACTGAGTACCGGAG-3(F) and 5-ACGAGCCATTTCTCCTTCA-3(R); GAPDH, 5-ACCTGACCTGTCTAGAA-3(F) and 5-TCCACCACCTGTTGCTGTA-3(R). The relative expression of indicated mRNAs was normalized to GAPDH.

2.7 | Cell invasion assay

Sixty microliters of Matrigel (Becton Dickinson) was added into the center of each chamber (Millipore). The cells were seeded in the upper chamber of the insert with or without drugs, after incubation for 24 hours at 37°C with 5% CO₂. The upper surface of the insert was scraped and the cells on the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet solution (Becton Dickinson). Cells in the bottom of the membrane were counted using a light microscope.

2.8 | Measurement of cell migration

Cells were planted into six-well plates to create a confluent monolayer with up to 80% cell confluence, and then starved for 24 hours with serum-free medium, followed by scratching with a sterile 200 μ L tip to manually create a wound. The cells were washed with PBS and cultured in medium supplemented with NAC, gefitinib or a combination of both. Images were acquired by inverted optical microscope after creating the wound.

2.9 | Measurement of apoptotic rate

Apoptotic cells were determined by Annexin V-FITC/propidium iodide (PI) Kit according to manufacturer's guidelines. The specific process referred to our previous work.²⁴ Briefly, PC-9/GR cells were treated with NAC, gefitinib or a combination of both for 48 hours, respectively. Afterward, cells were harvested, and stained with Annexin V-FITC and PI according to manufacturer's recommendations (Beyotime Institute of Biotechnology).

2.10 | Statistical analysis

The data were represented as mean \pm standard deviation (SD) or 95% confidence interval (CI) for three independent experiments. The GraphPad Prism software (version 5.0, GraphPad Software) was used for statistical analysis. The comparison between two independent treatment groups was analyzed by unpaired, two-tailed Student's *t* test. One-way analysis of variance (ANOVA) was utilized to analyse the variance among multi-sample. Statistical significance was assumed at $P < .05$.

3 | RESULTS

3.1 | Determination of dose-response curves and PC-9/GR EMT phenotype characteristics

Similar to our previous findings, the IC₅₀ value of PC-9/GR for gefitinib was 7.711 μ mol/L (95% CI: 7.058-9.657 μ mol/L;

Figure S1A) which was increased 141-fold compared with that of PC-9 cells (IC₅₀: 0.05471 μ mol/L, 95% CI: 0.04378-0.06835 μ mol/L; Figure S1B). This result showed a highly resistant effect to gefitinib in PC-9/GR cells. As presented in Figure S1C, E-cadherin was expressed in PC-9 cells, but downregulated in PC-9/GR cells. In addition, vimentin was upregulated in PC-9/GR cells, which was absent in PC-9 cells. These results demonstrated an EMT phenotype characteristic of PC-9/GR cells. The IC₅₀ of NAC in PC-9/GR cells was 15.53 mmol/L (95% CI: 14.50-16.62 mmol/L; Figure S1D).

3.2 | CalcuSyn-based analysis of NAC and gefitinib combination treatment

The constant combination ratio experiments were carried out at an equipotency ratio approximating their individual IC₅₀ (IC₅₀_{NAC}: IC₅₀_{gefitinib} \approx 2:1), which made sure the effect of each drug in combination was roughly equal. Figure 1A showed the dose-response curves for PC-9/GR cells exposed to NAC, gefitinib and both. CI values of the group treated with a combination of both drugs in different fractional cell growth inhibition (Fa) were shown in Figure 1B. CI values of less than 1 were acquired from the combination group, demonstrating that the two drugs must have a synergistic effect on growth inhibition. Then, PC-9/GR cells were treated with 5 mmol/L of NAC adding different concentrations of gefitinib. We found that the IC₅₀ of gefitinib was 0.3986 μ mol/L in the combination group, which was lower than gefitinib alone ($P < .001$; Figure 1C).

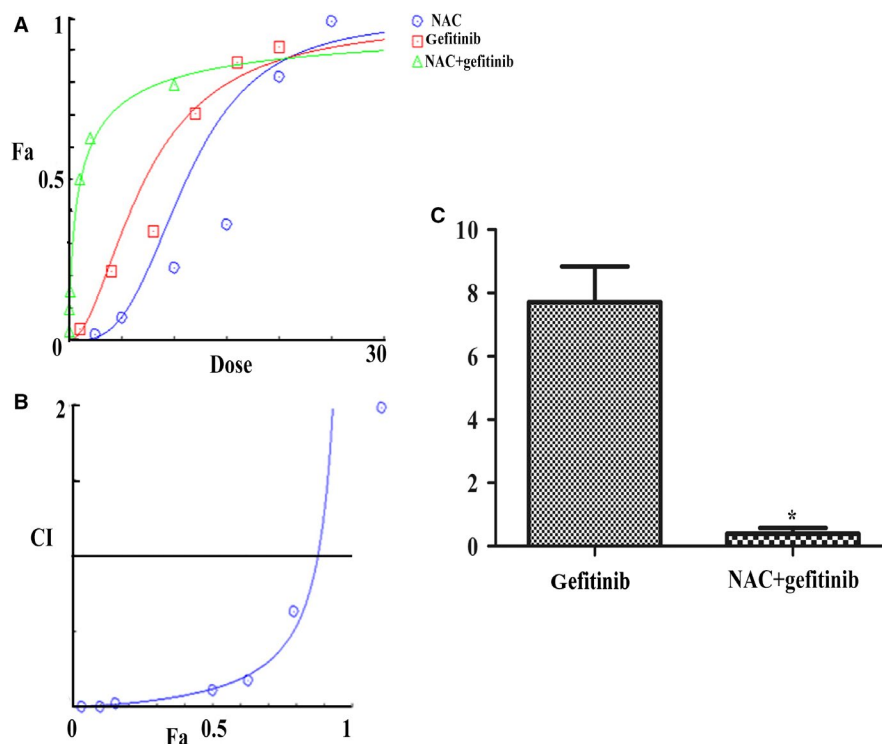


FIGURE 1 CalcuSyn-based analysis of the N-acetylcysteine (NAC) and gefitinib combination. A, Analysis of synergistic effect between NAC and gefitinib. B, CI values at different levels of growth inhibition effect. C, IC₅₀ of gefitinib in alone group and combination group. * $P < .001$ between NAC + gefitinib and gefitinib; Fa: fraction affected; IC₅₀: half maximal inhibitory concentration; CI: combination index

3.3 | Combination of NAC and gefitinib inhibited migration and invasion of PC-9/GR cells

To investigate whether NAC (5 mmol/L) in combination with gefitinib (2 μ mol/L) had an impact on biological behavior of PC-9/GR cells, we performed migration and invasion assays. After 48 hours of treatment with NAC or gefitinib alone or in combination (NAC + gefitinib group), the number of cells passing through the Matrigel decreased in the NAC + gefitinib group compared to that in either alone group (Figure 2A). Cell migration assay showed that the distance of cell migration was the shortest in the NAC + gefitinib group (Figure 2B). These data illustrated that NAC in combination with gefitinib could inhibit the invasion and migration of PC-9/GR cells.

3.4 | NAC in combination with gefitinib promoted apoptotic rate in PC-9/GR cells

Furthermore, we detected the apoptosis of PC-9/GR cells under different treatments using flow cytometry analysis.

NAC + gefitinib caused more apoptotic cells compared with NAC or gefitinib alone did ($P < .01$; Figure 3A,B). Bax and Bcl-2 are known as pro-apoptotic and anti-apoptotic molecules, respectively. The protein level of Bcl-2 was decreased in the NAC + gefitinib group. While treatment with NAC or gefitinib alone led to higher expression of Bax (Figure 3C). The trend of protein level was observed for Bcl-2 and Bax in the combination group compared to the other groups. These results demonstrated that NAC in combination with gefitinib promoted apoptosis of PC-9/GR cells.

3.5 | NAC in combination with gefitinib reversed EMT and inhibited Src activation in PC-9/GR cells

We next explored the underlying mechanism that led to the superior efficacy of NAC in combination with gefitinib. Western blot analysis showed that NAC in combination with gefitinib facilitated E-cadherin expression and inhibited

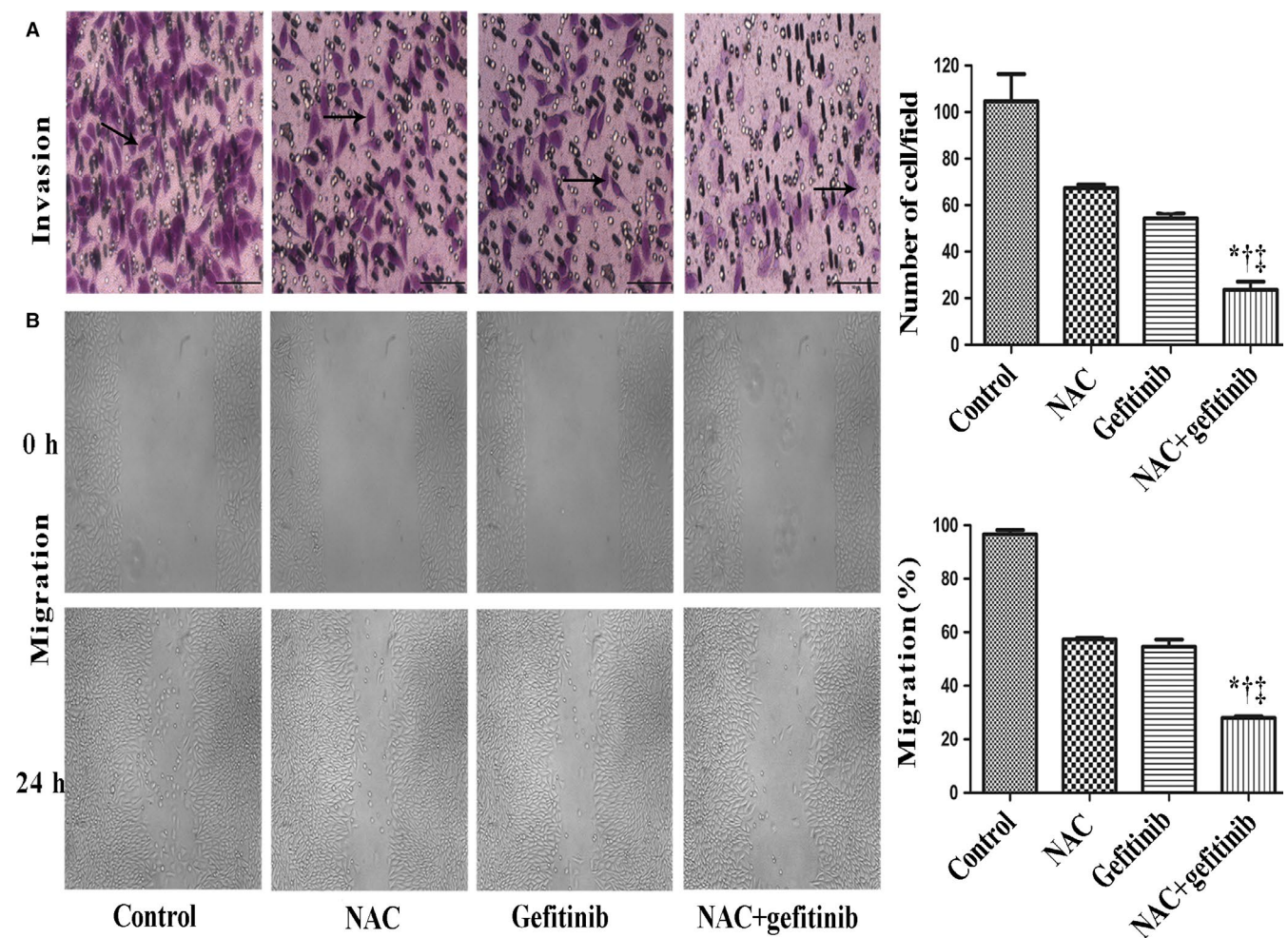


FIGURE 2 CalcuSyn-based analysis of the N-acetylcysteine (NAC) and gefitinib combination. A, Cells were pretreated with NAC or gefitinib alone and combination, PC-9/GR cells passed through the matriged was lower than other groups. B, Wound healing assays showed that the distance of cell migration in different group for 0 h, 24 h. NAC + gefitinib vs control, $*P < .001$; NAC + gefitinib vs NAC, $^{\dagger}P < .01$; NAC + gefitinib vs gefitinib, $^{\ddagger}P < .01$. Scale bars: 100 μ m. NAC, 5 mmol/L; gefitinib, 2 μ mol/L

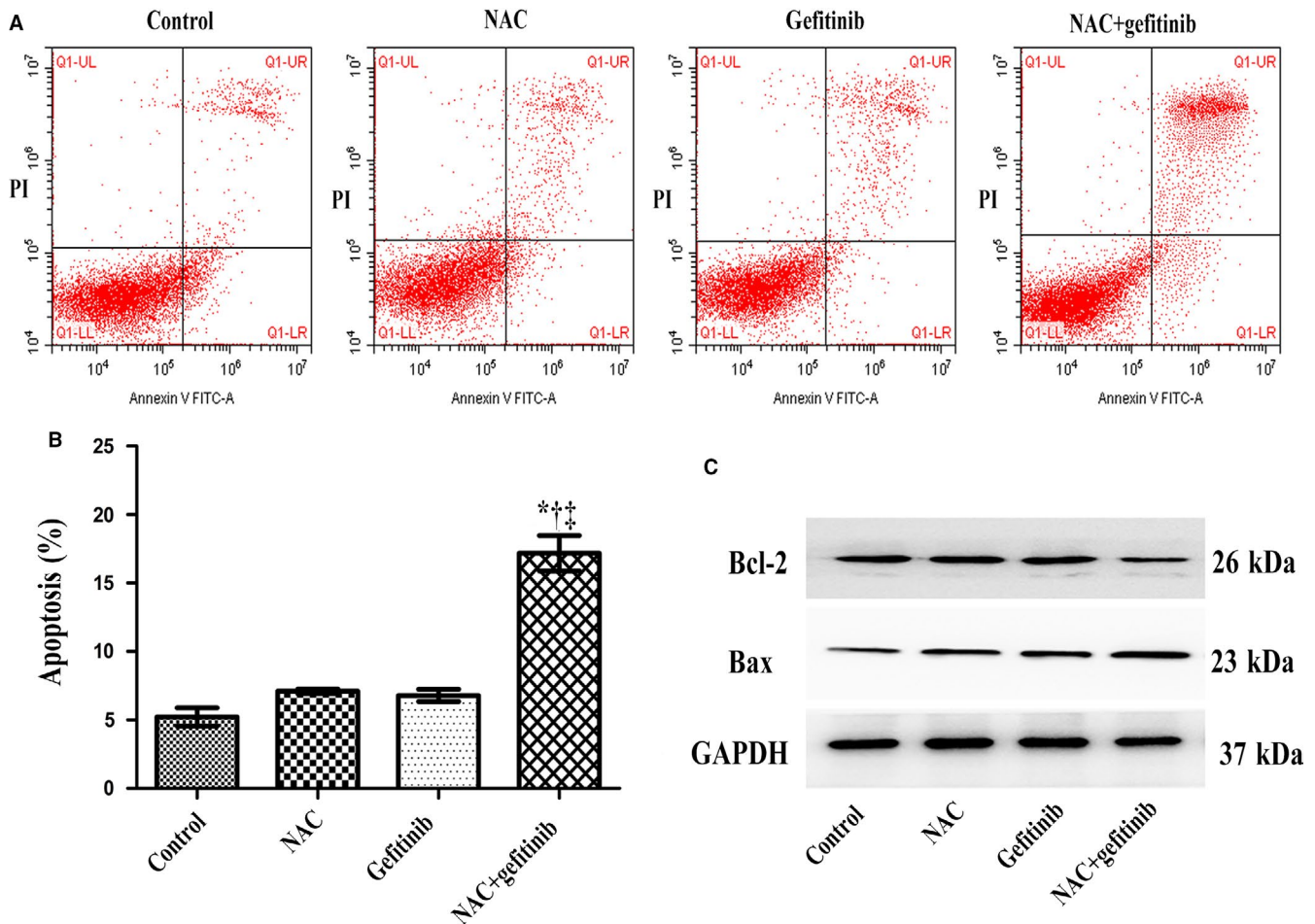


FIGURE 3 N-acetylcysteine (NAC) in combination with gefitinib induced PC-9/GR cells apoptosis. A and B, NAC combination with gefitinib induced apoptotic. C, The level of Bcl-2 protein expression was low, while Bax was high expression. NAC + gefitinib vs control, $*P < .001$; NAC + gefitinib vs NAC, $^{\dagger}P < .01$; NAC + gefitinib vs gefitinib, $^{\ddagger}P < .01$

Vimentin expression in PC-9/GR cells. In addition, the protein level of p-Src was decreased in the combination group (Figure 4A). In qRT-PCR analyses, expression of the E-cadherin increased and vimentin was decreased in the NAC + gefitinib group (Figure 4B). Then, we treated PC9/GR with PP2 (a potent inhibitor of Src, 10 $\mu\text{mol/L}$) in combination with gefitinib, which displayed the similar results as NAC + gefitinib did (Figure 4C,D). These results indicated that NAC in combination with gefitinib could inhibit Src activation and reverse EMT.

4 | DISCUSSION

NSCLC patients with EGFR mutations initially get good responses to EGFR-TKIs. However, these patients gradually acquire drug resistance inevitably. Therefore, it is of necessity to develop novel strategies to delay or overcome the acquired resistance to EGFR-TKIs. One of the interesting approaches would be the combination treatment with alternative drug in

addition to gefitinib. Drug combination is widely used and has become the primary treatment modality for cancer.²⁶ A combination of gefitinib with metformin shows a synergistic effect and increases sensitivity of patients with lung cancer to gefitinib.²⁷ In this study, we used NAC in combination with gefitinib, which could effectively overcome drug resistance to gefitinib.

NAC is a precursor of glutathione, a powerful antioxidant used in anti-tumor research. It has been reported that NAC inhibits proliferation and invasive behavior of human cancer cells in vitro, including colorectal cancer, bladder cancer, prostate cancer, tongue cancer, and lung carcinoma.²⁸⁻³¹ Our previous study showed that NAC could overcome gefitinib resistance induced by cigarette smoke extract. NAC has been proven to inhibit the growth of lung carcinomas by reducing cell proliferation and facilitating apoptosis in tobacco carcinogen-treated A/J mice.³² NAC exerts inhibitory effect on tumor growth via modulation of EGFR/AKT signaling and HBP1 expression in EGFR-overexpressed oral cancer.³³ It can be inferred that NAC

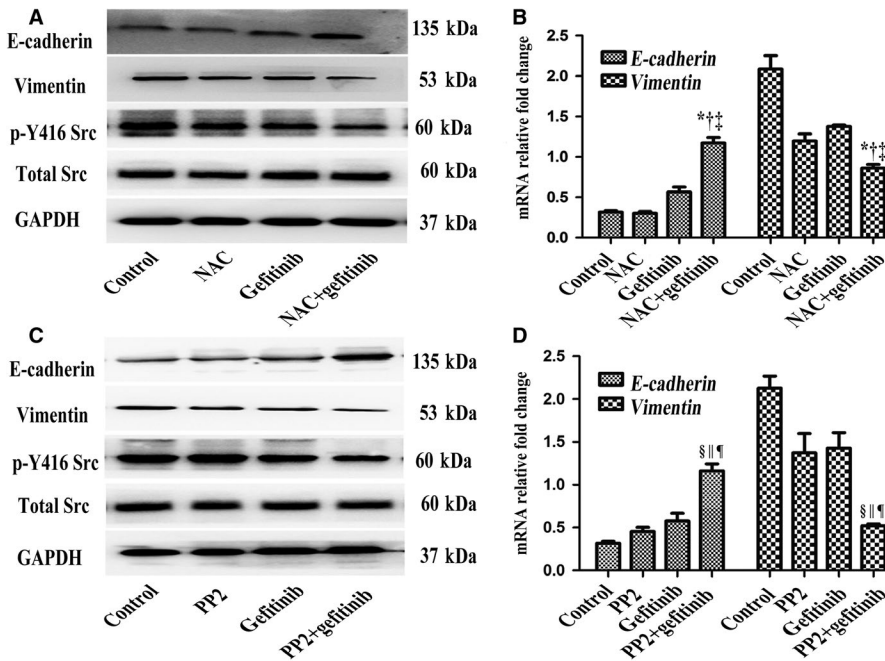


FIGURE 4 N-acetylcysteine (NAC) in combination with gefitinib reversed EMT and inhibited Src activation in PC-9/GR cells. A and B, NAC combination with gefitinib could inhibited Src activation and reversed EMT. C and D, PP2 combination with gefitinib could inhibit Src activation and reverse EMT in PC-9/GR cells. NAC + gefitinib vs control, $*P < .001$; NAC + gefitinib vs NAC, $^{\dagger}P < .01$; NAC + gefitinib vs gefitinib, $^{\ddagger}P < .01$; PP2 + gefitinib vs control, $^{\S}P < .001$; PP2 + gefitinib vs NAC, $^{\parallel}P < .01$; PP2 + gefitinib vs gefitinib, $^{\P}P < .01$. PP2, 10 $\mu\text{mol/L}$

has promising potential to be a novel anticancer agent. In this study, we demonstrated that NAC could overcome gefitinib resistance by inhibiting Src activation and reversing EMT in PC-9/GR cells when used in combination with gefitinib. It has been demonstrated that Src is a key regulator of EMT in cancer cells.^{34,35} Our research verified that NAC modulated EMT of lung cancer cells by inhibiting the activation of Src, which might clarify the underlying mechanism theoretically.

EMT is one of hallmarks in cancer and plays a crucial role in the development and progression of most solid tumors.³⁶ As a candidate mechanism of drug resistance, EMT plays a crucial role in acquired EGFR-TKIs resistance as reported by different research groups.^{37,38} It has been demonstrated that EMT could be regarded as a predictor of therapy response in patients with NSCLC. Furthermore, there is a direct connection between EMT and EGFR-TKIs sensitivity. Research has shown that E-cadherin potentiates the sensitivity to gefitinib to improve the effect.³⁹ Notably, EMT co-occurs with EGFR T790M mutations in a study using re-biopsies.⁴⁰ Multiple signaling networks modulate EMT, such as transforming growth factor- β 1 (TGF- β 1), mitogen-activated protein (MAP) kinases, RHOA, AKT, and STAT3. Multiple molecules are involved in EMT, including Src, a key oncogene.

Src is the first oncogene ever identified, and its family members have also been recognized as potential targets in cancer therapy. Activation of Src promotes numerous pathological processes, including invasion, migration, proliferation, and angiogenesis in a variety of cancers.⁴¹ Increased Src activity boosts EMT process, while Src inhibition suppresses this process. Moreover Src-mediated EMT is involved in the chemotherapy resistance of cancers.^{42,43} Some studies concentrate on the combination of EGFR inhibitors and Src

inhibitors. A previous study has revealed that the efficacy of Src inhibitors combined with EGFR inhibitors is synergistic, and Src inhibitors could improve gefitinib resistance in NSCLC with EMT.⁴⁴ Our previous studies have shown that Src is involved in cigarette smoke-induced EMT and EGFR-TKI resistance, and Src inhibition sensitizes resistant cells to gefitinib. However, further investigations are needed to determine whether this combination in vivo could delay drug resistance and prolong patient progression-free survival (PFS) and overall survival (OS).

A lack of in vivo study is an obvious limitation in the current study, and we will conduct in vivo experiments in animal models to verify the cytological results in this study. We have not further investigated the up-and-down pathway of apoptotic proteins, which is another limitation of this study. To prove the synergistic effect of this combination therapy for EGFR mutant lung cancer, the future study should demonstrate with other EGFR mutant lung cancer cell lines. In addition to gefitinib, osimertinib has obtained positive results from the phase 3 AURA trial and phase 3 FLAURA trial.^{45,46} With the approval of osimertinib in Nov 2015 (<https://www.fda.gov/>), it will be clinically meaningful if osimertinib has the similar effect when combined with NAC.

In conclusion, we used the combination index value to evaluate the efficacy of NAC and gefitinib combination treatment. Our results demonstrated that NAC had synergistic effect with gefitinib. These two drugs used in combination overcame the resistance of PC-9/GR to gefitinib by inhibiting Src activation and reversing EMT. Thus, these findings highlighted a novel insight into overcoming gefitinib resistance and provided a potential strategy for NSCLC patients.

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CONFLICT OF INTEREST

We declare no conflicts of interest in association with this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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