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Original Article

WZ4003 sensitizes non-small cell lung cancer cells to gefitinib via inhibition of ARK5 and epithelial-to-mesenchymal transition

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Abstract: Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, is used as a first-line treatment for advanced non-small cell lung cancer (NSCLC); however, its utility is hampered by the development of chemoresistance. This study aimed to investigate the synergistic role of WZ4003, a novel (nua) kinase (NUAK) inhibitor, in enhancing gefitinib sensitivity in NSCLC cells. Our data indicated WZ4003 enhances the sensitivity of NSCLC cells to gefitinib. We also found ARK5 knockdown in NSCLC cell lines increased their sensitivity to gefitinib. However, WZ4003 did not affect gefitinib sensitivity when ARK5 was knocked down in NSCLC cell lines (using siRNA). Both WZ4003 and ARK5 inhibition suppressed epithelial-to-mesenchymal transition by reducing the expression of vimentin and increasing E-cadherin expression. Together, our results demonstrate WZ4003 plays a vital role in releasing acquired resistance to gefitinib by inhibiting ARK5 and epithelial-to-mesenchymal transition. Therefore, synergistic use of WZ4003 and gefitinib may prevent the development of gefitinib resistance in NSCLC.

Keywords: Non-small cell lung cancer (NSCLC), gefitinib, WZ4003, epithelial to mesenchymal transition (EMT)

Introduction

The mortality rate of non-small-cell lung cancer (NSCLC) is increasing worldwide. Surgery remains the first choice of therapy for patients with NSCLC [1]; however, many are diagnosed at advanced stage, where other interventions are required. Unfortunately, despite growing progress in the management of NSCLC, the prognosis of advanced NSCLC remains poor, with a 5-year overall survival rate of ~11% [2-4]. In such advanced cases of NSCLC, gefitinib, an epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), is used as a first-line therapeutic agent [5, 6]. Although many patients with NSCLC respond positively to primary treatment with gefitinib, most will eventually develop chemoresistance [7, 8]. Thus, the potential mechanism(s) underlying gefitinib resistance in NSCLCs must be explored, and new therapeutic options are required.

The novel (nua) kinase (NUAK) subfamily includes two members: the NUAK family SNF1-like kinase-1 (NUAK1) or AMPK-related kinase 5 (ARK5), and NUAK2 or the sucrose non-fermenting AMPK-related kinase (SNARK) [9]. The NUAK subfamily belongs to the AMP activated protein kinase (AMPK) family, which is activated by the liver kinase B1 (LKB1) tumor-inhibiting protein kinase. Accumulating evidence demonstrates ARK5 and NUAK2 are involved in the regulation of cell adhesion, cancer cell invasion, embryonic development, senescence, proliferation, neuronal polarity, and axon branching [9-12]. As ARK5/NUKA2 inhibition has been shown to suppress the malignant characters of cancers, inhibitors of NUAK kinases may be useful for the treatment of some tumors [13-15]. WZ4003 was shown to be a highly specific NUAK kinase inhibitor when compared to 139 other protein kinases [16]. This compound was found to markedly suppress the NUAK1-

mediated phosphorylation of myosin phosphate-targeting subunit 1 (MYPT1) [16]. WZ4003 was also reported to have similar effects to NUAK1 knockout in mouse embryonic fibroblasts and NUAK1 knockdown in a U2OS cell line in terms of inhibiting cell migration, invasion, and proliferation [16, 17]. However, little is known about the role of WZ4003 in cancer cells, especially NSCLC.

In the present study, we aimed to explore the role of WZ4003 in gefitinib resistance and uncover the potential mechanisms. Our results for the first time demonstrated that wz4003 could improve gefitinib resistance in NSCLC cells via inhibiting ARK5 and EMT process.

Materials and methods

Cell culture and reagents

NSCLC cell lines (NCL-H1299, HCC827, A549) were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). HCC827 was cultured in DMEM (Gibco, USA), while NCL-H1299 and A549 were maintained in RPMI1640 (Gibco, USA). All culture media were supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. Gefitinib and WZ4003 were obtained from Selleck (Houston, TX, USA) and dissolved in DMSO.

Transfection

The ARK5 siRNA and negative siRNA was designed and synthesized by Genepharm (Shanghai, China). In brief, 50 nM siRNA and Lipofectamine 2000 reagent mixture (Invitrogen, Carlsbad, CA, USA) prepared in DMEM basic medium was added to the cells cultured in 6-well plates for 36 or 48 h for interference before harvesting the cells for further experiments.

CCK-8 assay

HCC827 cells (7,000 cells/well), NCL-H1299 (3,000 cells/well), or A549 cells (3,000 cells/well) were seeded into 96-well plates. After the cells had adhered, the culture medium (without 10% FBS) was replaced for 24 h before exposing the cells to the various treatments (for 48 h). Then, the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) was used to examine the cell viability at 1-3 h according to the manufacturer's instructions. An MRX II microplate reader (Dynex, Chantilly, VA, USA) was used to measure the OD value at 450 nm.

Western blot analysis

Western blot was performed as described elsewhere. The primary antibodies used were as follows: ARK5 (Abcam, Cambridge, MA, USA), E-cadherin (Abcam), Vimentin (Abcam), and GAPDH (Cell Signaling Technology, Beverly, MA, USA); all antibodies were used at a dilution of 1:1000. Proteins were visualized with corresponding horseradish peroxidase (HRP)-coupled secondary antibody from Cell Signaling Technology (1:5000 dilution). Membranes were washed three times with TBST (Tris-buffered saline, 0.1% Tween 20) for 10 min each time. The enhanced chemiluminescence method was used to visualize target protein bands.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time PCR analyses were performed with the SYBR Premix ExTaq kit (RR420A, Takara, Dalian China), with GAPDH used as the internal control gene. ABI 7500 was used to perform the qRT-PCR assays, and the relative expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method. The forward and reverse primers for ARK5 were: ARK5, Forward 5'-CCTCATTCGGCAAATCAGCA-3'. ARK5, Reverse 5'-GGGTTCACCATCAGCATCCAC-3'.

EDU assay

Proliferation of the NSCLC cell lines (NCL-H1299, HCC827, A549) were determined using the Click-iTEdU Imaging kit (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol. The cell nuclei were coloured blue, and EDU positive cells were colored green.

Statistical analysis

SPSS16.0 software was used for all statistical analyses. The experimental data are represented as mean ± standard deviation (SD), and analyzed by a two-tailed Student T test and one-way analysis of variance (ANOVA), Two-way ANOVA and Bonferroni's post hoc test were applied to assess the effects of gefitinib and the combined treatment. IC50s were calculated using non-liner regression by GraphPad Prism version 6.0. Statistical significance was accepted if $P<0.05$.

Results

WZ4003 enhances gefitinib sensitivity in NSCLC cells

First we determined the effects of WZ4003 on the viability of NSCLC cells using the CCK-8 assay. We found WZ4003 at concentrations above 2.5 μM reduced the viability of NSCLC cells (**Figure 1A**). To examine the synergistic effects of WZ4003 and gefitinib, we examined the viability of NSCLC cell lines that were pretreated with 1.25 μM WZ4003 for 6 h and then exposed to different concentrations of gefitinib. We showed NSCLC cells pretreated with WZ4003 were more sensitive to gefitinib than those treated with gefitinib alone (**Figure 1B**). We then confirmed that combined WZ4003 and gefitinib treatment significantly enhances the anti-proliferative effect of gefitinib in NSCLC cell lines using the EDU assay (**Figure 1C**, **1D**).

ARK5 expression is associated with the gefitinib response in NSCLC cells

As WZ4003 is a specific inhibitor of ARK5 and ARK5 is reported to be involved in chemoresistance in NSCLC [18], we hypothesized the function of WZ4003 on regulating gefitinib sensitivity is dependent on ARK5. Therefore, we examined the expression of ARK5 in the three lung cancer cell lines. ARK5 expression was highest in the NCL-H1299 cell line, followed by the A549 cell line, then the HCC827 cell line (**Figure 2A**, **2B**). Next, we examined the gefitinib sensitivity of the three NSCLC cell lines and found gefitinib sensitivity increased as ARK5 expression levels decreased (**Figure 2C**). Indeed, NSCLC cells with higher ARK5 expression levels had higher IC50 values for gefitinib (**Figure 2D**).

ARK5 knockdown increases gefitinib sensitivity in NSCLC cells

To further investigate the function of ARK5 in regulating gefitinib sensitivity in NSCLC cells, we used ARK5 siRNA to knockdown its expression. We found ARK5 inhibition enhanced sensitivity to gefitinib in a dose-dependent manner using the CCK-8 assay (**Figure 3A**), which was confirmed in the EDU assay (**Figure 3B**, **3C**). The interfering efficiency of ARK5 was verified by Western blot (**Figure 3D**). This suggests ARK5 is a key regulator of the development of gefitinib resistance.

WZ4003 sensitizes NSCLC cells to gefitinib via ARK5

To verify whether WZ4003 regulates gefitinib resistance via ARK5, we examined the viability of NSCLC cells treated with either ARK5 siRNA alone or with ARK5 siRNA plus 1.25 μM WZ4003 for 48 h, when cultured under different concentrations of gefitinib. We found the cell viability and IC50 values of gefitinib were similar between the two groups (**Figure 4A**, **4B**), indicating WZ4003 is not effective when ARK5 levels are low. This phenomenon was also observed in our EDU analysis (**Figure 4C**, **4D**). The interfering efficiency of ARK5 was verified by Western blot (**Figure 4E**). This data proves ARK5 is involved in the WZ4003-mediated increase in gefitinib sensitivity.

WZ4003 and ARK5 knockdown regulates the expression of EMT-associated proteins

As epithelial-to-mesenchymal transition (EMT) is important in the acquisition of drug resistance in many solid tumor cells [19, 20], we hypothesized EMT may be involved in the WZ4003-mediated regulation of gefitinib sensitivity. We found both WZ4003 treatment and ARK5 knockdown lead to changes in the expression of EMT-associated proteins in all three NSCLC cell lines tested (**Figure 5**). In particular, WZ4003 treatment and ARK5 knockdown increased the expression of E-cadherin and reduced expression of vimentin. This suggests the EMT process is inhibited by WZ4003 and ARK5 inhibition.

Discussion

Gefitinib has proven to be clinically effective in the treatment of NSCLC, but its use is still limited due to acquired resistance [21]. As ARK5 and NUAK2 have previously been shown to play essential roles in tumor cell survival, invasion, and metastasis [13, 22, 23], we examined whether a NUAK kinase inhibitor (WZ4003) could be a useful anti-cancer agent in NSCLC. Although the efficacy of WZ4003 has previously been confirmed [12, 24], the function of WZ4003 in NSCLC was unclear, until now.

The novel kinase (NUAK) subfamily includes two members, NUAK1 or AMPK-related kinase 5 (ARK5) and NUAK2 or sucrose nonfermenting AMPK-related kinase (SNARK), which is associ-

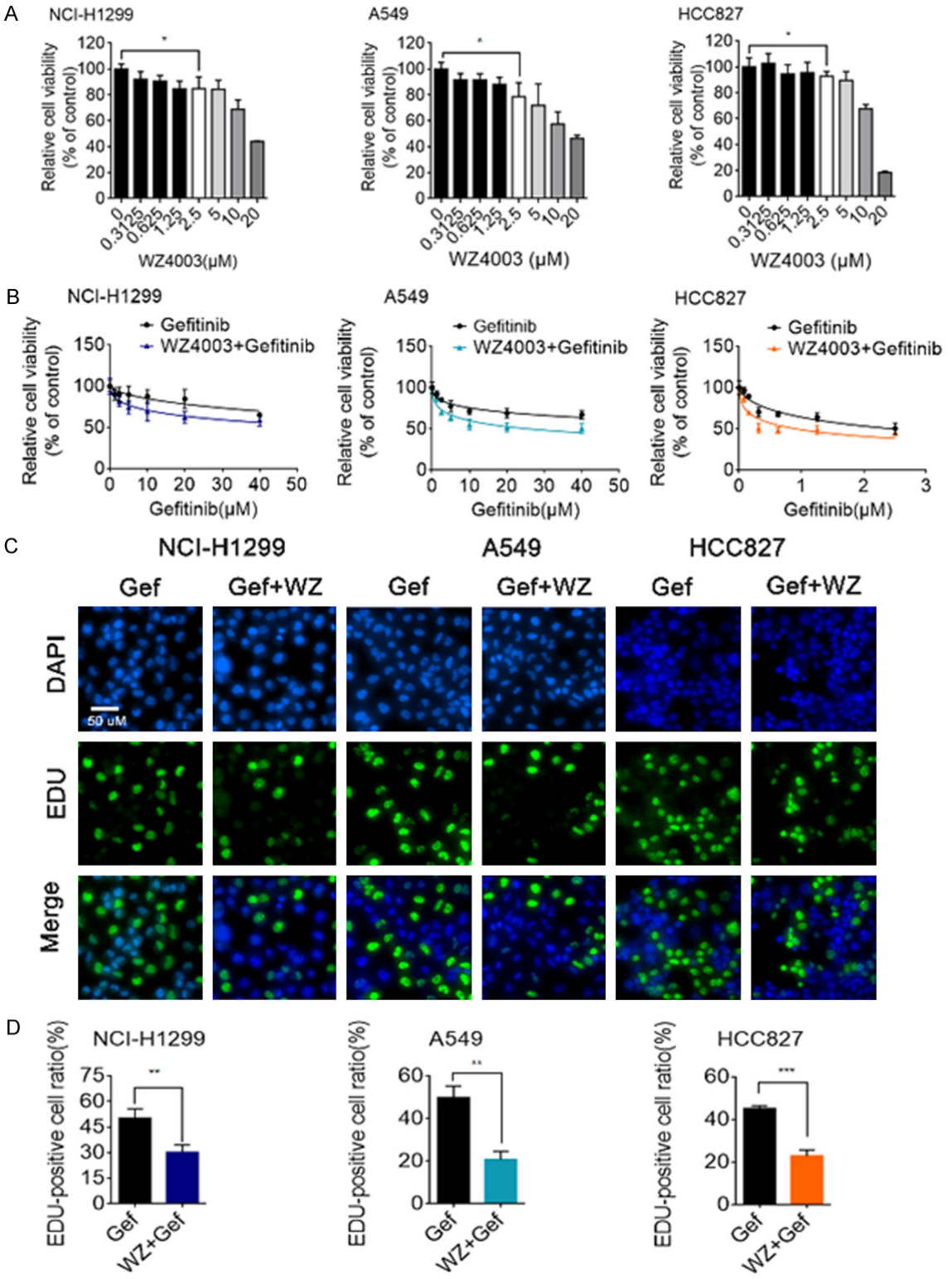

Figure 1. WZ4003 enhances gefitinib sensitivity in NSCLC cells. A. Viability of NSCLC cells exposed to different concentrations of WZ4003 for 24 h using the CCK-8 assay. The percentage of cell viability is shown relative to untreated controls. B. Viability of NSCLC cells treated in different concentrations of gefitinib with or without 2.5 μM WZ4003 for 24 h using the CCK-8 assay. Bars represent mean ± SD from at least three independent experiments. C, D. Cells were treated with 50 μM gefitinib combined with or without 2.5 μM WZ4003 for 24 h. Cell proliferation was subsequently examined by the EDU assay. The EDU positive cells were counted in five random views and averaged. (*P<0.05, **P<0.01, ***P<0.001).

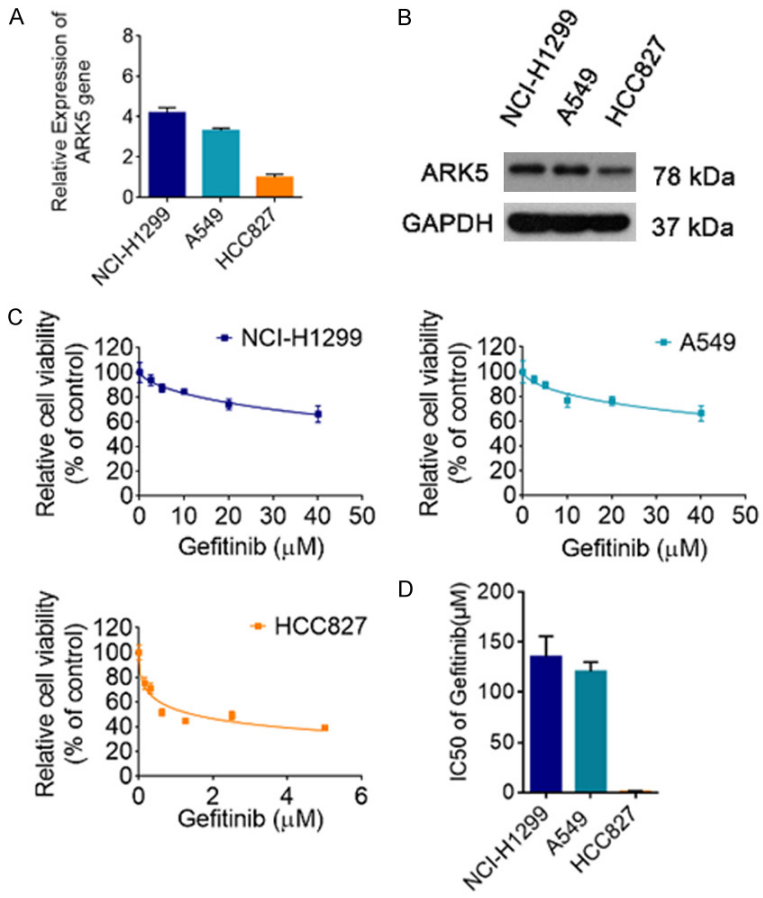

Figure 2. ARK5 expression levels and gefitinib sensitivity in NSCLC cells. (A) mRNA and (B) protein expression (western blot) of ARK5 in the three NSCLC cell lines (GAPDH was used as a loading control). ARK5 expression was highest in the NCL-H1299 cell line. (C) Viability of NSCLC cells detected under different concentrations of gefitinib using the CCK-8 assay. (D) The IC50 value was calculated based on the CCK-8 results.

ated with protein metabolism, polarity, and cellular homeostasis [9]. Indeed, abnormal expression of ARK5 and NUAK2 have previously been found in several cancers, including breast, gastric, liver, colorectal, and pancreatic cancer [25-29]. In addition, both ARK5 and NUAK2 have been shown to play an essential role in regulating malignant behaviors, including tumor cell survival, invasion, and metastasis [13, 22, 23]. Considering the important role of NUAK kinases in cancers, it proposed that NUAK kinase inhibitors may play a effective anti-cancer role. WZ4003 was proved as high selected NUAK kinase inhibitors, and did not significantly inhibit the activity of 139 other protein kinases [16]. The efficiency of WZ4003 was proved in other two studies [12, 24]. However, up to now, no reports have demonstrated the function of WZ4003 in NSCLC. In the present, we revealed WZ4003 enhances gefitinib sensitivity in NSCLC via inhibiting ARK5 and EMT. Our findings are in line with those of a previous study, which showed ARK5 can regulate EMT and was a potential target for reverting chemoresistance in NSCLC [18]. And our results was partly consisted with the previous study.

EMT is a complicated process, resulting in the loss of cell adhesion proteins, which can induce epithelial cells to transform into mesenchymal phenotypes and lead to increased invasion, migration, and chemoresistance [30, 31]. Accumulating reports demonstrate EMT is related to carcinogenesis, metastasis, and chemoresistance in many tumors including NSCLC [19, 20]. Furthermore, EMT plays a key role in the development of gefitinib resistance in NSCLC [32-34]. Our data indicate that WZ4003 and ARK5 inhibition may reduce levels of the mesenchymal marker, vimentin, and increase expression of the epithelial marker E-cadherin. This suggests WZ4003 might play a vital role in EMT and thus in modulating gefitinib sensitivity of NSCLC cells.

In conclusion, WZ4003 may enhance the gefitinib sensitivity of NSCLC cells via inhibiting EMT and ARK5. These findings provide novel insight into overcoming gefitinib resistance. In particular, the combined use of WZ4003 and gefitinib may be overcome chemoresistance and enhance the clinical effect of gefitinib in NSCLC.

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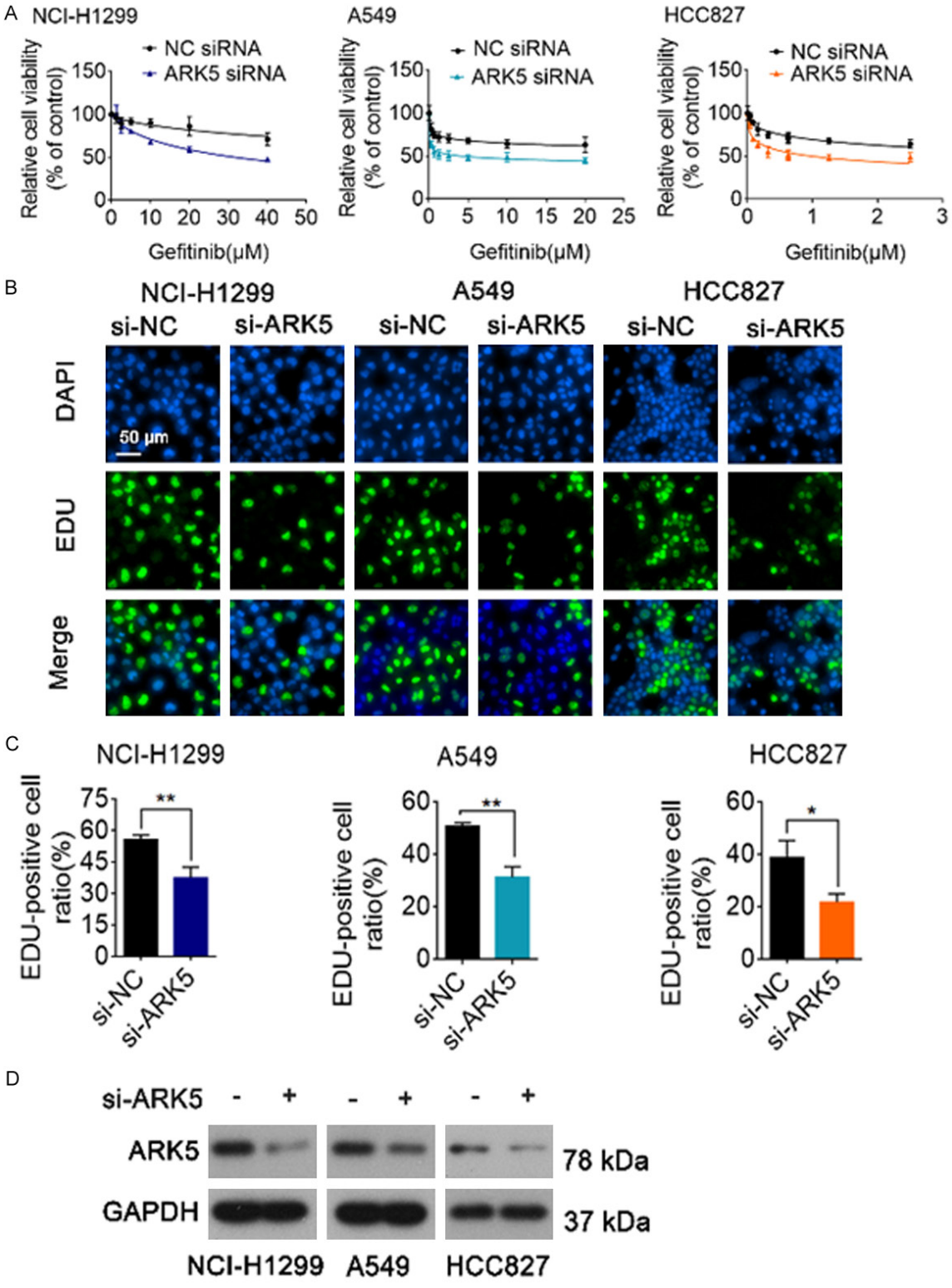

Figure 3. ARK5 knockdown increases gefitinib sensitivity in NSCLC cells. A. Viability of NSCLC cells with or without ARK5 knockdown detected under different concentrations of gefitinib using the CCK-8 assay. B, C. Proliferation of NSCLC cells with or without ARK5 knockdown under 50 μM gefitinib using the EDU assay. The EDU positive cells were counted, bars represent mean ± SD from at least three independent experiments. D. The efficiency of ARK5 knockdown by siRNA as verified by western blot; GAPDH was used as the internal control. (**P<0.01, ***P<0.001).

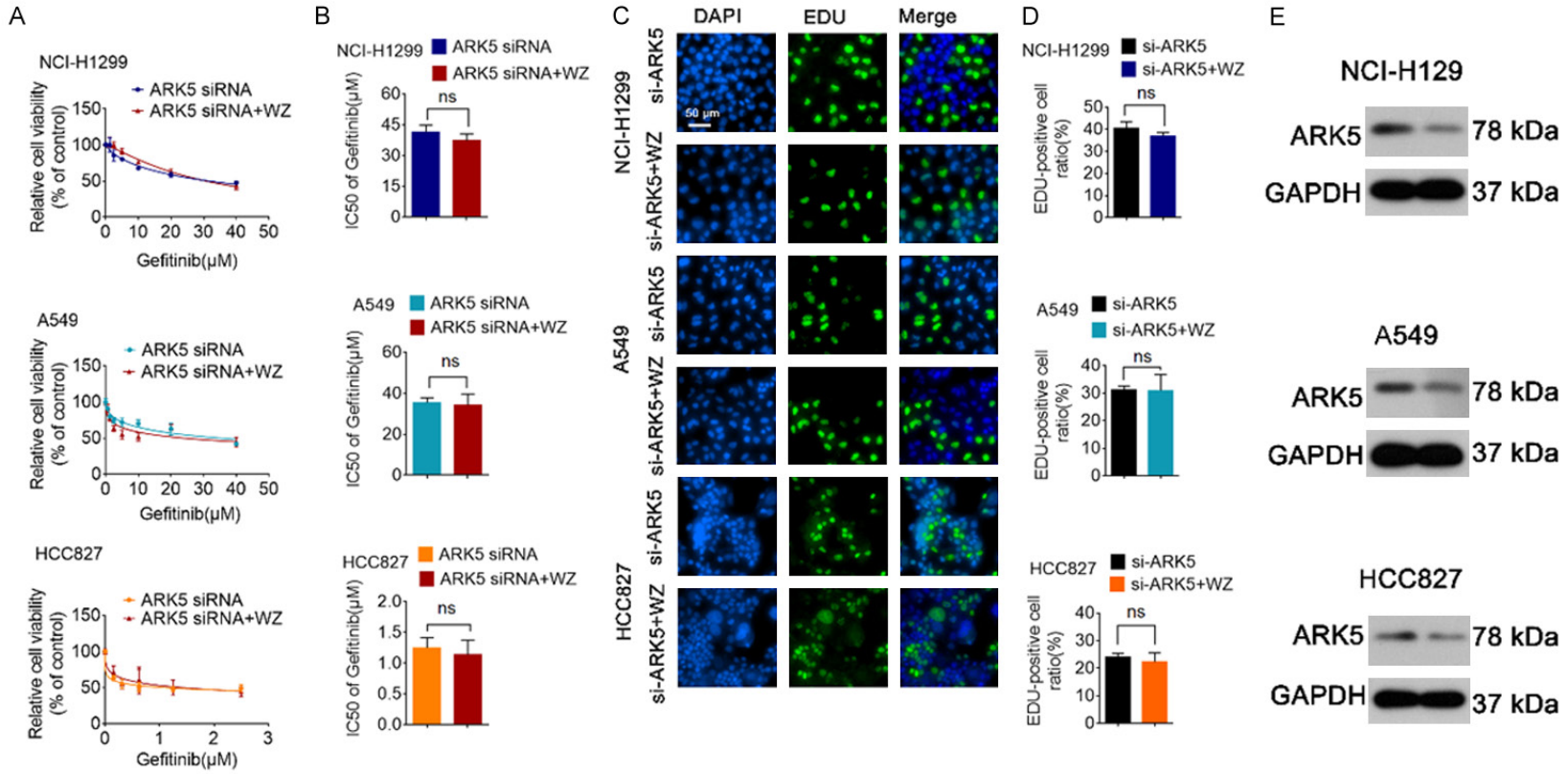

Figure 4. ARK5 knockdown alleviates the WZ4003-mediated increase in gefitinib sensitivity. A, B. Viability of NSCLC cells treated with ARK5 siRNA or ARK5 siRNA plus 2.5 μM WZ4003 under different concentrations of gefitinib using the CCK-8 assay. The IC50 value was calculated based on the CCK-8 results. C, D. Proliferation of NSCLC cells treated with ARK5 siRNA or ARK5 siRNA plus 2.5 μM WZ4003 under different concentrations of gefitinib using the EDU assay, The EDU positive cells were counted, bars represent mean ± SD from at least three independent experiments. E. The efficiency of ARK5 knockdown by siRNA as verified by western blot; GAPDH was used as the internal control. (ns, no significance).

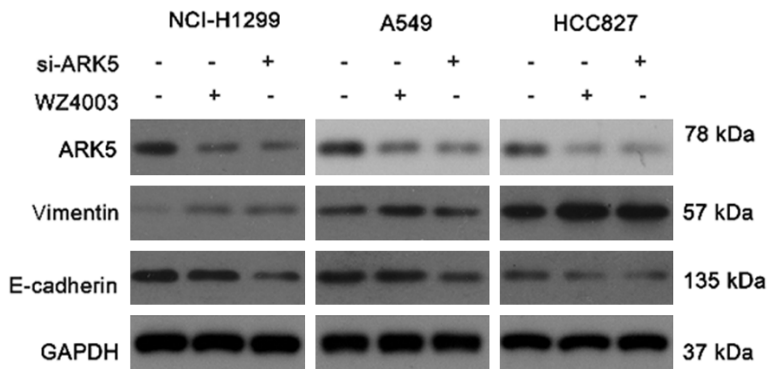

Figure 5. WZ4003 and ARK5 knockdown reduces the expression of EMT-associated proteins. Western blot analysis of the expression of E-cadherin and vimentin in NSCLC cells with ARK5 knockdown or treated with 2.5 μM WZ4003 or the negative control (NC); the results were quantified by comparing with GAPDH.

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Disclosure of conflict of interest

None.

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