

## Original Article

# Inhibition of AMPK-related kinase 5 (ARK5) enhances cisplatin cytotoxicity in non-small cell lung cancer cells through regulation of epithelial-mesenchymal transition

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**Abstract:** Lung cancer incidence and mortality rates are amongst the highest of all malignant tumors worldwide. ARK5 is a member of the human AMP-activated protein kinase (AMPK) family which is implicated in tumor survival and progression. The current study was designed to explore the role of ARK5 in resistance of non-small cell lung cancer (NSCLC) to cisplatin. We studied the sensitivity of two NSCLC cell lines, NCI-H1229 and A549, to cisplatin by using proliferation and cell viability assays. We then examined expression of ARK5, Twist, and the epithelial to mesenchymal transition (EMT) biomarkers, E-cadherin and Vimentin, by Western blot and immunofluorescence. We found that ARK5 downregulation significantly increased the cisplatin chemosensitivity of NSCLC cells, and that NCI-H1229 cells, which express high levels of ARK5 and possess a mesenchymal phenotype, were more resistant to cisplatin than A549 cells, which show low expression ARK5. Furthermore, siRNA-mediated silencing of ARK5 resulted in altered EMT patterns in NSCLC cells. These data support a role for ARK5 in regulating EMT in NSCLC cells. Together, our findings suggest that ARK5 is a potential drug target for combating drug resistance and regulating EMT in NSCLC cells.

**Keywords:** ARK5, non-small cell lung cancer (NSCLC), cisplatin, epithelial-mesenchymal transition (EMT), drug resistance

## Introduction

Lung cancer has one of the highest incidence and mortality rates of all malignant tumors globally, and accounts for approximately one third of all cancer-related deaths [1, 2]. Approximately 20% of lung cancers are small cell lung cancers (SCLCs) and 80% are classified as non-small cell lung cancers (NSCLCs), which have a 5-year survival rate that is less than 5% when accompanied by metastasis [3]. At present, chemotherapy is one of the most effective treatments for NSCLCs, and cisplatin is the standard first-line chemotherapy drug for NSCLCs; however, long term therapeutic effects are diminished by chemoresistance [4]. Although there are many factors inducing chemoresistance in NSCLC therapy, the mechanisms of cisplatin resistance remain unclear.

Epithelial-mesenchymal transition (EMT), a process wherein epithelial cells depolarize, lose their cell-cell contacts, and gain an elongated, fibroblast-like morphology, is a potential mechanism by which tumour cells gain metastatic features [5]. Many studies have shown that EMT may be a key process regulating resistance to chemotherapy in malignant tumors, with functional implications of EMT including enhanced mobility, invasion, and resistance to apoptotic stimuli [6-9]. There are some reports that NSCLCs with an epithelial phenotype are more sensitive to chemotherapy than those with a mesenchymal phenotype and that the expression of E-cadherin in tumors with a mesenchymal phenotype restores chemosensitivity [3]. In addition, EMT is associated with acquisition of malignant and stem-cell characteristics [10].

ARK5 is a novel member of the human AMP-activated protein kinase (AMPK) family, which was discovered to be key molecules mediating migration activity of human pancreatic cancer cells [11]. It is directly phosphorylated and activated by Akt and has been found to induce tumor cell survival during nutrient starvation in an Akt-dependent manner [12]. In addition, ARK5 is a key mediator of the actions of Akt which play a very important in cancer cell proliferation, survival, tumorigenesis, invasion and metastasis [13-15], and it is a tumor survival and tumor progression factor [16]. A recent study demonstrated that ARK5 activation by Akt could enhance tumor invasion and metastasis in pancreatic cancer cells [17]. Similar results have also been reported in colorectal cancer and breast cancer [11, 12]. Furthermore, ARK5 has recently been shown to be upregulated in ovarian cancer tissues compared with adjacent normal tissues, and has been demonstrated to promote EMT, and activation of ARK5 may be positively associated with EMT in ovarian cancer [16]. However, this process has not been completely elucidated in NSCLC cell lines.

In this study, we examined the role of ARK5 in NSCLC cell lines, and found that A549 cells, which show low expression of ARK5, were more sensitive to cisplatin than NCI-H1229 cells which show high levels of ARK5 expression. Knockdown of ARK5 enhanced the sensitivity of NSCLC to cisplatin. Additionally, we confirmed that ARK5 promoted EMT in NSCLC cell lines. Thus, activation of the ARK5 may be positively associated with EMT in NSCLC cell lines.

### Materials and methods

#### *Cell culture*

The human NSCLC cell lines (A549 and NCI-H1229) were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA). All cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, USA) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Cells were maintained under 5% CO<sub>2</sub> at 37°C in a humidified incubator.

#### *siRNA transfections*

NSCLC cells were transfected with ARK5 siRNA (100 nM) or Twist siRNA (100 nM; Santa Cruz

Biotechnology, Dallas, TX, USA) using Lipofectamine 2000 according to the manufacturer's protocol. The transfection medium was replaced with complete medium 6 h after transfection, and the cells were incubated for the indicated times. All treatments were started 24 h after transfection.

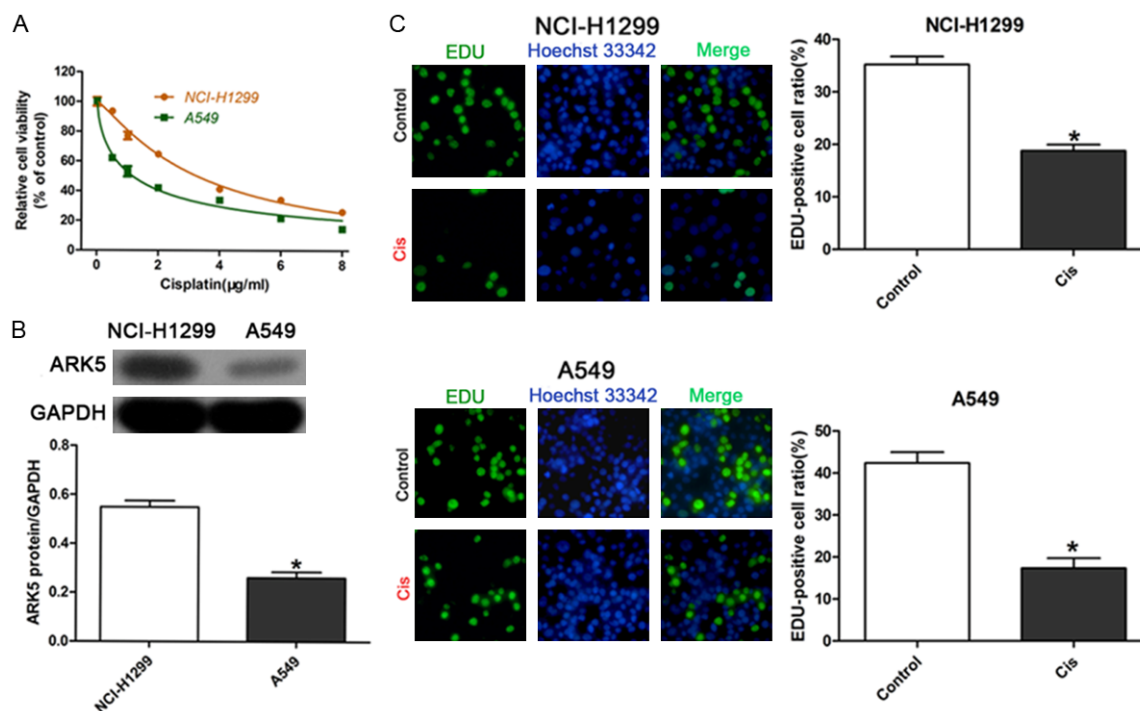
#### *Cell viability assay*

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to measure relative cell viability after NSCLC cell treatment. NSCLC cells (3 × 10<sup>3</sup> cells/well) were seeded in 96-well plates and cultured for 24 h. The culture medium was replaced by 10% FBS-medium containing the drug concentration indicated. After a further incubation for 48 h, 10 µL of CCK-8 solution was added, cells were incubated for an additional 3 h, and then absorbance at 450 nm was measured using an MRX II microplate reader (Dynex Technologies, Chantilly, USA). Relative cell viability was calculated as a percentage of untreated controls.

#### *Western blot*

For extraction of proteins, the cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors (Sigma, St. Louis, MO, USA) was added to NSCLC cells collected after the treatment for 48 h in an ice bath, and samples centrifuged at 1200 rpm for 5 min at 4°C after the lysis treatment. The supernatant was collected and the protein concentrations measured with a BCA Protein Assay Kit (Sigma, St. Louis, MO, USA). The protein samples (40 µg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), blocked with Tris-buffered saline (TBS) and 0.1% Tween 20 (TBS-T) containing 5% bovine serum albumin, and incubated overnight at 4°C with primary antibody (E-cadherin, Vimentin, Twist or ARK5, diluted 1:1000 in TBS-T). The membrane was washed three times with TBS-T, and then the appropriate horseradish peroxidase-labeled secondary antibody (1:2000) was incubated 2 h at room temperature. The protein bands were detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Data were quantified by the optical density of each band and GAPDH was used as an internal control.

# Inhibition of ARK5 enhances cisplatin cytotoxicity in NSCLC



**Figure 1.** Expression of ARK5 may be associated with cisplatin resistance. A. Sensitivity of A549 and NCI-H1299 cells to. Cell viability was measured following a 48 h treatment using a CCK-8 assay. B. Western blot showing ARK5 expression in the NSCLC cell lines. GAPDH served as a loading control. C. Images and quantification of EdU staining of NSCLC cells with (Cis) or without (control) cisplatin treatment. \*P < 0.05. All experiments were performed at least three times.

## 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

NSCLC cell lines were seeded in 96-well plates at a density of  $3 \times 10^3$  cells/well in growth media. The medium was replaced with serum-free medium to synchronize the cells. After 24 h, the serum-free medium was replaced with growth media containing drugs at the indicated concentrations for 48 h. Cell proliferation was assessed using an a Click-iTEdU imaging kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

## Immunofluorescence

NSCLC cells were seeded into 48-well plates at  $3 \times 10^3$  cells/well. Cells were fixed with 4% formaldehyde for 15 min, washed with PBS, treated with 5% BSA for 30 min at room temperature, and incubated with anti-E-cadherin (1:200) or anti-human Vimentin (1:200) primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The cells were incubated with FITC-conjugated secondary antibody (Abcam, Cambridge, USA) at 4°C for 2 h. Nuclear staining was performed with DAPI

(Sigma, St. Louis, MO, USA) at room temperature for 2 min. Following two washes with PBS, cells were observed using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

## Statistical analysis

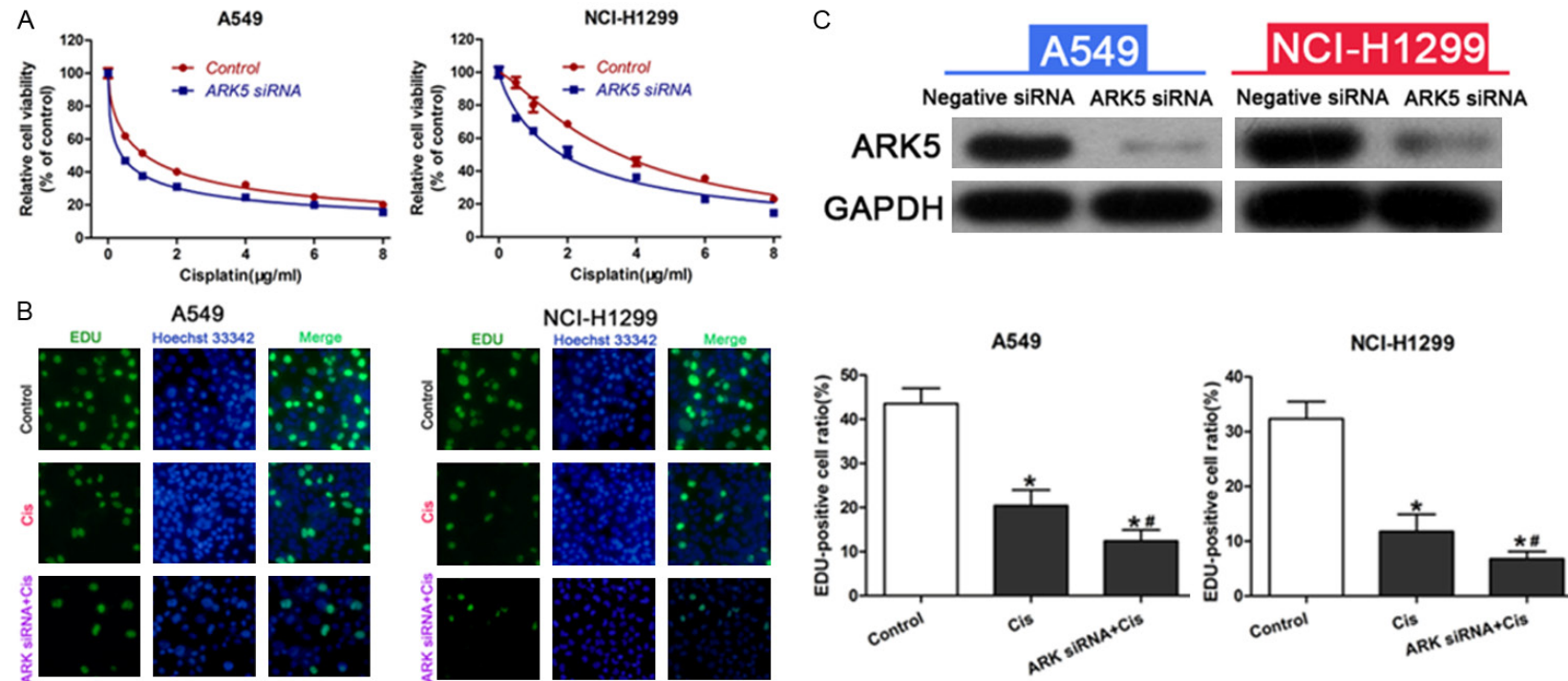
Data are presented as the mean  $\pm$  SD and analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) and the SPSS software package (Version 18.0, Chicago). Statistical differences between two groups were examined with the Student's t test and multiple groups were compared using one-way analysis of variance (ANOVA). A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### ARK5 expression may contribute to the sensitivity of NSCLC cell lines to cisplatin

To assess the cytotoxicity of cisplatin in NSCLC cells, we used a CCK-8 assay to measure cell viability. Compared with the NCI-H1299 cell line, A549 cells were more sensitive to cisplatin (Figure 1A). Western blotting was used to mea-

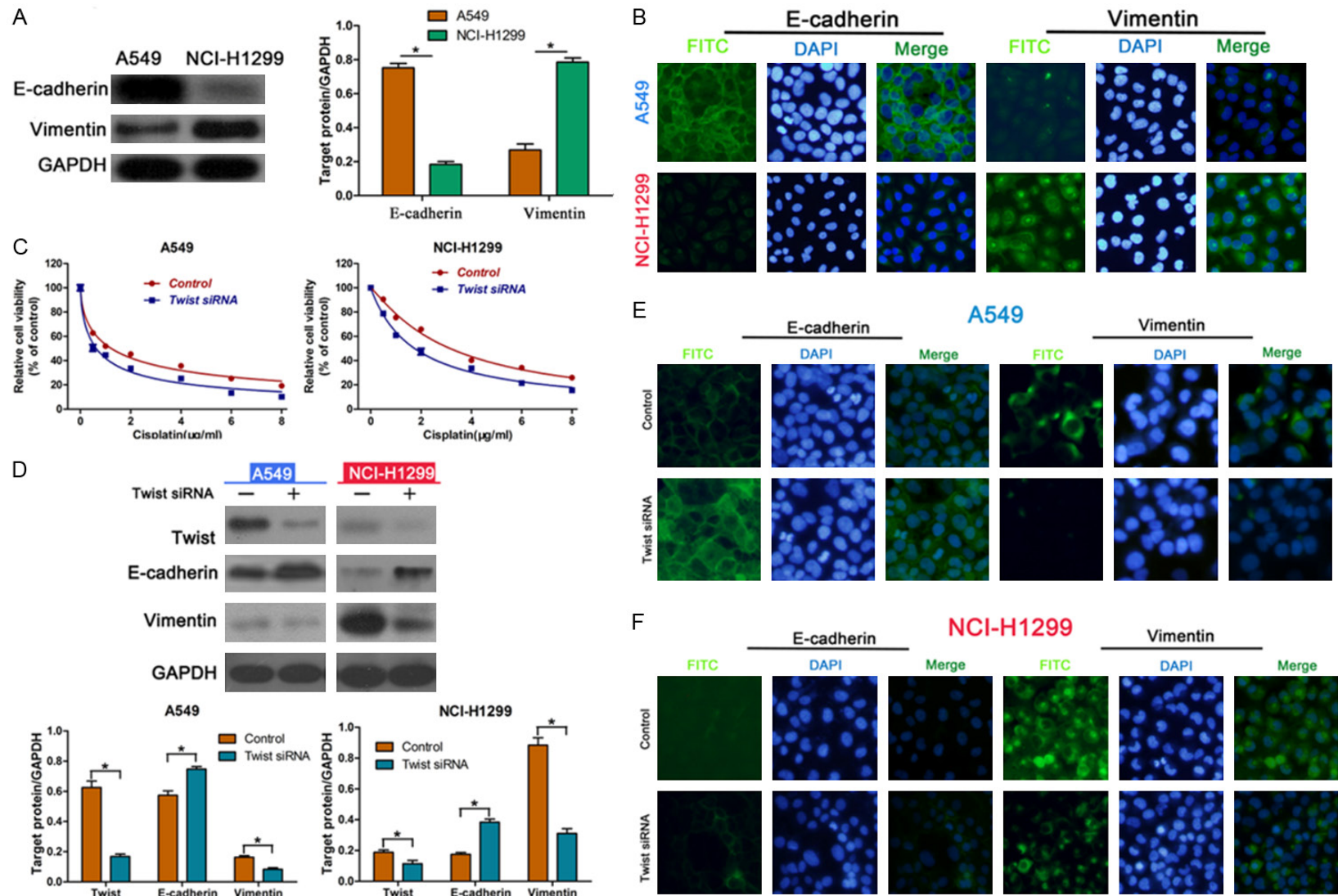
## Inhibition of ARK5 enhances cisplatin cytotoxicity in NSCLC



**Figure 2.** Knockdown of ARK5 enhance the cisplatin sensitivity of A549 and NCI-H1299 cells. **A.** Cell viability assay demonstrating that cisplatin sensitivity is increased in A549 and NCI-H1299 cells following siRNA knockdown of ARK5. **B.** Images and quantification of EdU staining following knockdown of ARK5 and treatment with cisplatin for 48 h. \*P < 0.05. **C.** Western blot showing ARK5 expression in NSCLC cells transfected with ARK5 siRNA or a negative control siRNA following treatment with cisplatin for 48 h.



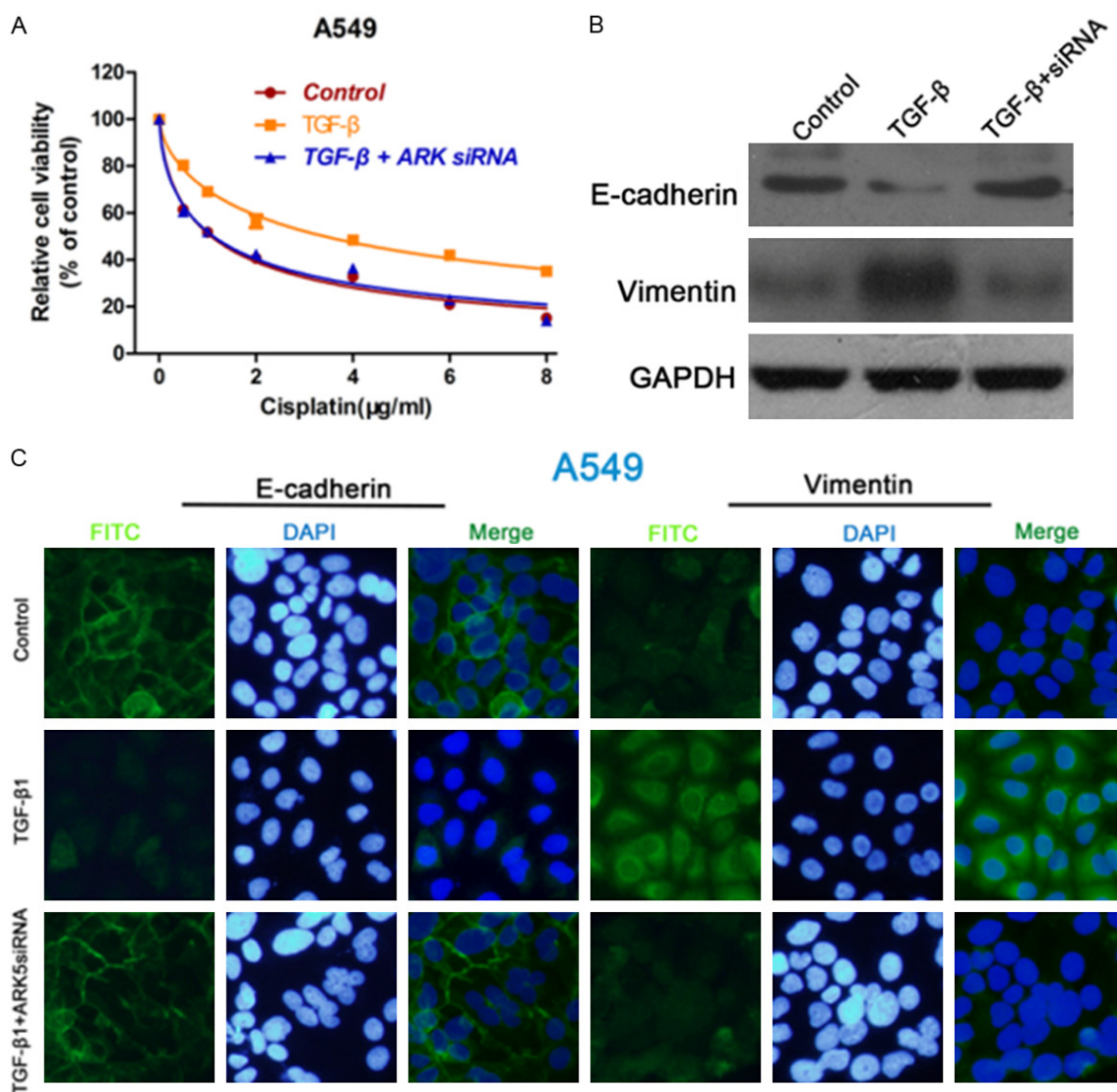
# Inhibition of ARK5 enhances cisplatin cytotoxicity in NSCLC



**Figure 3.** Knockdown of Twist enhances sensitivity to cisplatin. **A.** Western blot showing the expression levels of E-cadherin and Vimentin in NSCLC cells. **B.** Immunofluorescence assay showing the expression levels of E-cadherin and Vimentin in NSCLC cells. \*P < 0.05. All experiments were performed at least three times. **C.** Knockdown of Twist enhances cisplatin sensitivity of A549 and NCI-H1299 cells. **D.** Western blot showing Twist, E-cadherin and Vimentin expression in NSCLC cells transfected with Twist siRNA or a negative control siRNA and treated with cisplatin for 48 h. Relative protein expression in NSCLC cells was quantified by band density with GAPDH serving as a loading control. \*P < 0.05. All experiments were performed at least in triplicate. **E.** Immunofluorescence analysis of expression

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of E-cadherin and Vimentin in A549 cells transfected with Twist siRNA or a negative control siRNA. \*P < 0.05. All experiments were performed at least three times. F. Immunofluorescence analysis of expression of E-cadherin and Vimentin in NCI-H1299 cells transfected with Twist siRNA or a negative control siRNA. \*P < 0.05. All experiments were performed at least three times.



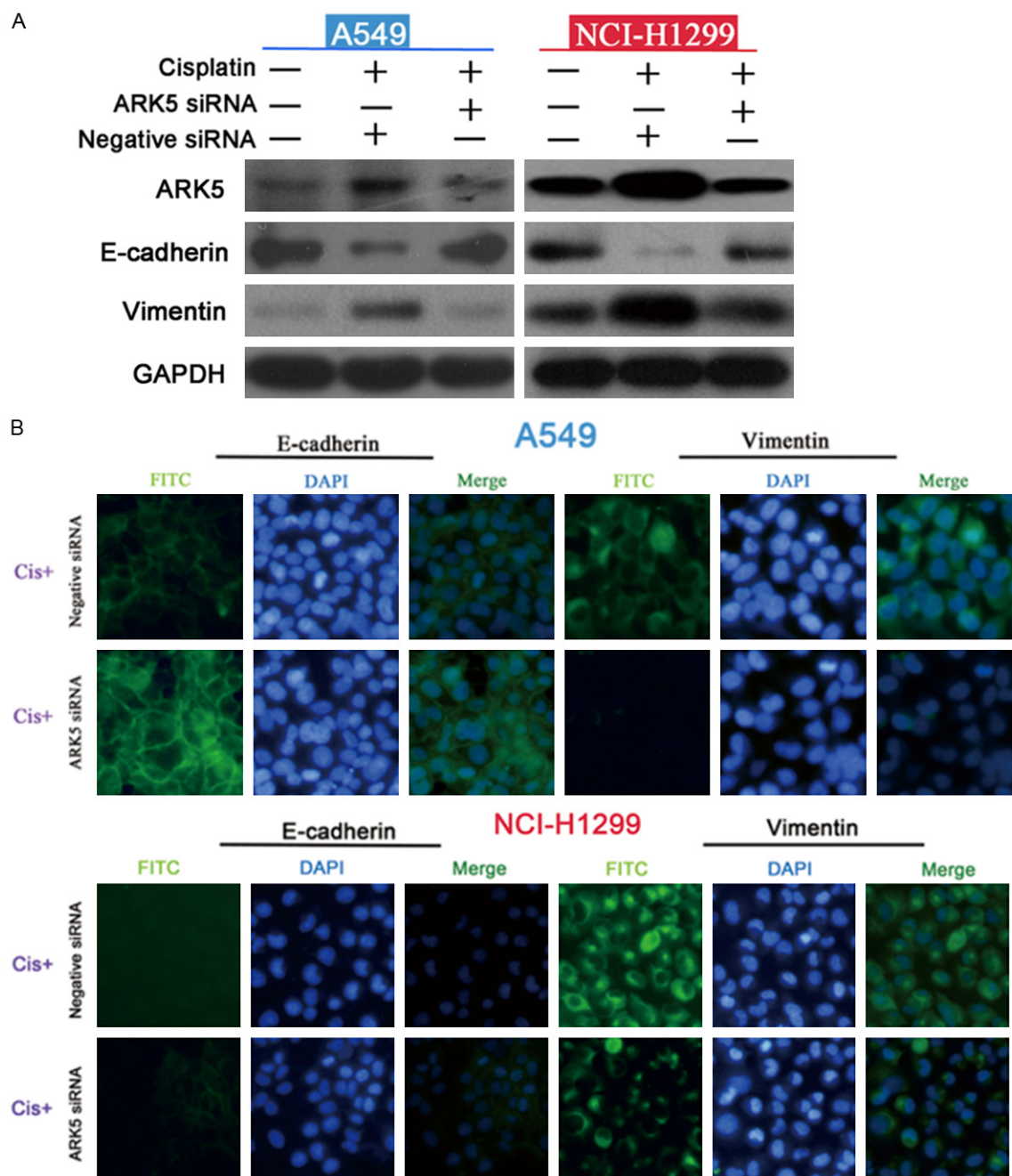
**Figure 4.** Inhibition of ARK5 increases cisplatin sensitivity. A. The cell viability of A549 under TGF-β1 or both TGF-β1 and ARK5 siRNA, control was under normal condition. Cell viability was measured using CCK-8 method. B. Western blot was used to detect the expression of E-cadherin and Vimentin in A549 under TGF-β1 or both TGF-β1 and ARK5 siRNA, GAPDH was as control protein. C. Immunofluorescence was performed to analyze the expression of E-cadherin and Vimentin in A549 cells under TGF-β1 or both TGF-β1 and ARK5 siRNA, control was under normal condition. \*P < 0.05. All experiments were performed at least three times.

sure the expression of ARK5 in NSCLC cells. Interestingly, we observed higher expression of ARK5 in NCI-H1299 cells than in A549 cells (Figure 1B), indicating that ARK5 may be involved in the chemoresistance to cisplatin. EdU incorporation assay was used to test the effects of cisplatin on proliferation of NSCLC cells following a 48 h treatment. As shown in

Figure 1C, proliferation of NSCLC cells was reduced after treatment with cisplatin.

*Knockdown of ARK5 enhances cisplatin sensitivity of A549 and NCI-H1299 cells*

We hypothesized that the expression of ARK5 could affect cisplatin sensitivity in NSCLC cells. To test this, we transfected ARK5 siRNA into

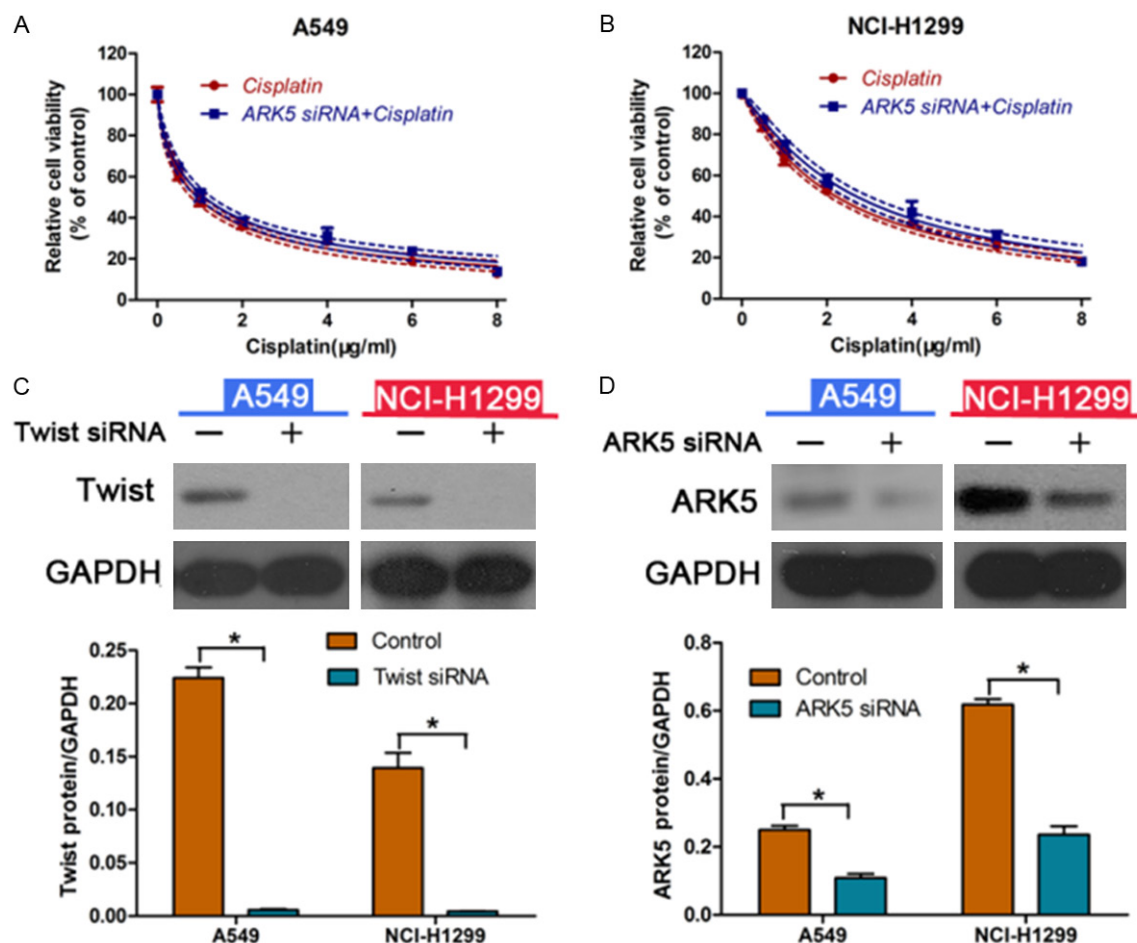


**Figure 5.** ARK5 knockdown prevents EMT during cisplatin treatment. A. Western blot showing E-cadherin and Vimentin expression with ARK5 siRNA and/or cisplatin treatment. \* $P < 0.05$ . All experiments were performed at least three times. B. Immunofluorescence images showing expression of E-cadherin and Vimentin following ARK5 siRNA and cisplatin treatment in NSCLC cells.

NSCLC cells (Figure 2A). We then performed a CCK-8 assay to assess cell viability following treatment with cisplatin, and found that ARK5 siRNA significantly improved cisplatin-induced growth inhibition in both cell lines. In addition, an EdU incorporation assay was used to detect cell proliferation. As shown in Figure 2B, com-

pared with the cisplatin group, the proliferation of A549 and NCI-H1299 cells was reduced after knockdown ARK5 and treatment with cisplatin. Knockdown was confirmed by Western blot (Figure 2C). These results demonstrate that inhibiting ARK5 significantly enhances the effects of cisplatin in NSCLC cells.





**Figure 6.** Knockdown of Twist enhances sensitivity to cisplatin. Cell viability assay quantification comparing A549 (A) and NCI-H1299 (B) cells treated with cisplatin following knockdown of Twist or ARK5. Knockdown efficiency of Twist (C) and ARK5 (D) by assessed by Western blot and quantified.

#### Twist knockdown promotes cisplatin sensitivity of A549 and NCI-H1299

In order to investigate the mechanism of ARK5-regulated sensitivity to chemotherapy, we examined whether EMT contributed to drug resistance [18, 19]. We used Western blotting to examine differential expression of phenotypic markers in NSCLC cells, and detected the expression of E-cadherin (epithelial phenotype) and Vimentin (mesenchymal phenotype) by immunofluorescence in NSCLC cells. The results indicated that, compared to NCI-H1299 cells, A549 cell showed higher expression of the epithelial marker E-cadherin and lower expression of the mesenchymal marker Vimentin. (Figure 3A, 3B). Twist is involved in the process of EMT, which plays an essential role in cancer metastasis [20]. Therefore, we assessed whether ARK5 could regulate resistance to cisplatin

in A549 and NCI-H1299 cells by interacting with Twist. Interestingly, we found that knockdown of Twist increased sensitivity to cisplatin in A549 and NCI-H1299 cells (Figure 3C). As shown in Figure 3D, Western blotting indicated that knockdown of Twist led to upregulation of E-cadherin and downregulation of Vimentin. Immunofluorescence staining also showed results consistent with the Western blot analysis (Figure 3E).

#### Inhibition of ARK5 reverses cisplatin resistance

Previous studies have shown that TGF-β1 can induce epithelial cells to undergo EMT. In this study, we treated cells with TGF-β1 (10 ng/mL for 48 h) to induce epithelial-type A549 cell to undergo EMT. We then investigated changes in both EMT marker expression and cisplatin sen-



sitivity. Western blot analysis showed that treatment with TGF- $\beta$ 1 could induce downregulation of the expression of E-cadherin and increased Vimentin expression. Knockdown of the expression of ARK5 in TGF- $\beta$ 1-treated cells reversed EMT, demonstrated by increased E-cadherin expression and reduced Vimentin expression (**Figure 4A**). We then performed a CCK-8 assay to measure the effects of TGF- $\beta$ 1 treatment on cisplatin chemosensitivity. Additionally, we knocked down expression of ARK5 and examined the effects of TGF- $\beta$ 1 treatment on cisplatin chemosensitivity. TGF- $\beta$ 1-induced mesenchymal A549 cells showed reduced cisplatin sensitivity, while knockdown of ARK5 in TGF- $\beta$ 1-treated cells increased cisplatin sensitivity (**Figure 4B**). Immunofluorescence staining also showed results consistent with the Western blot analysis (**Figure 4C**).

## *Inhibition of ARK5 reverses EMT of NSCLC cells during cisplatin treatment*

Although we determined that ARK5 could regulate expression of EMT markers, it was not clear whether this effect was associated with cisplatin treatment. We found that cisplatin-treated NSCLC cells showed upregulation of the expression of ARK5 and also showed EMT changes during treatment with cisplatin. These results suggest that ARK5 expression may contribute to cisplatin resistance. To further examine this effect, we transfected ARK5 siRNA into NSCLC cell and carried out cisplatin treatment. The results showed that cisplatin combined with ARK5 siRNA knockdown increased the expression of E-cadherin and decreased Vimentin expression, indicating that the NSCLC cells transferred an epithelial phenotype after downregulation of ARK5 (**Figure 5A**). Immunofluorescent staining also confirmed the similar results consistent with the Western blot analysis (**Figure 5B**).

## *ARK5 knockdown reverses EMT, regulating resistance of cisplatin*

Inhibition of ARK5 expression increased chemosensitivity of NSCLC cells to cisplatin. We next attempted to determine whether this phenotype resulted from inhibition of EMT. Previous studies in breast cancer cells have implicated Twist in drug resistance, and have suggested that EMT contributes to a resistant phenotype [19]. NSCLC line cells were transfected with

Twist siRNA and treated with cisplatin. We observed no differences as compared to knockdown of ARK5 when cells were treated with cisplatin (**Figure 6A, 6B**). Western blotting was performed to determine knockdown efficiency (**Figure 6C, 6D**). The results show that ARK5 could reverse EMT, enhancing the effects of cisplatin.

## **Discussion**

NSCLC is the most commonly diagnosed and fatal cancer worldwide. Chemotherapy serves as an important component of postoperative treatment for cancer patients [21]. However, drug resistance has become a significant challenge to successful chemotherapy [22]. At present, cisplatin is the most commonly used chemotherapeutic drugs for treating NSCLC [23]. Unfortunately, drug resistance negatively impacts the effects of this treatment. The mechanisms involved in cisplatin resistance remain unclear, so studies addressing this issue are needed.

ARK5 has been identified as the fifth member of the AMPK family. It is a key tumor cell survival factor that mediates Akt signaling. Akt is known to play a key role in tumor malignancy, including tumor cell survival, invasion, and metastasis [11]. Overexpression of ARK5, which is activated by Akt, has been found to markedly stimulate tumor cell invasion and metastasis, in breast cancer, ovarian cancer, squamous cell carcinoma, colorectal cancer, and pancreatic cancer [24-27]. To date, however, no reports have examined the role of ARK5 in NSCLC. It is very important for us to understand the molecular basis of ARK5 involvement in invasion and metastasis of NSCLC. As cisplatin resistance is an important factor contributing to NSCLC recurrence, we hypothesized that ARK5 could be involved in cisplatin resistance in NSCLC. Based on our Western blot and CCK-8 assay results, we found that the expression of ARK5 was inversely correlated with sensitivity to cisplatin. NCI-H1299 cells, which show high expression of ARK5, were more resistant to cisplatin than A549 cells that show low expression of ARK5. We transfected ARK5 siRNA into NSCLC cells to knockdown the expression of ARK5, and the results showed that ARK5 siRNA treatment resulted in increased sensitivity to cisplatin as compared

to the negative control. It is now well accepted that Twist, which may function as a multifunctional proto-oncogene during tumorigenesis and progression of solid tumors, protects cells from chemotherapy-induced apoptosis and senescence and promotes tumor EMT [28-30]. We showed that knockdown of Twist could enhance the effects of cisplatin chemotherapy. Furthermore, transfection of Twist siRNA or ARK5 siRNA followed by cisplatin treatment showed similar effects. These data show that ARK5 plays a major role in regulating cisplatin resistance in NSCLC cells.

EMT is a complex process associated with the loss of cell adhesion proteins that can induce epithelial cells to transform to a mesenchymal phenotype and result in increased invasion, migration, and cell proliferation [31]. Activation of EMT triggers tumor cell invasion and dissemination and is thus considered as the initiating step of cancer metastasis [32, 33]. Many studies have shown that EMT is related to carcinogenicity, metastasis and poor prognosis in many tumors including NSCLC [34-37], and it has been suggested that EMT is a determinant of drug resistance in NSCLC [38-40]. Our data indicates that knockdown of ARK5 reduces expression of the mesenchymal marker Vimentin, and increases expression of the epithelial marker E-cadherin during cisplatin treatment. NSCLC cells in which ARK5 has been knocked down also show increased sensitivity to cisplatin. TGF- $\beta$  signaling plays a major role in the EMT process and previous reports have been shown that TGF- $\beta$ 1 induces EMT in NSCLC [41]. Our data showed that treatment with TGF- $\beta$ 1 of the epithelial type A549 cells led to higher levels of Vimentin and lower levels of E-cadherin. However, EMT could be reversed if A549 cells were pre-treated with ARK5 siRNA. These results suggest that the expression of ARK5 might play an important role in EMT phenotype changes and modulate cisplatin chemotherapy sensitivity of NSCLC cells.

In this study, we established that ARK5 is an important factor contributing to cisplatin resistance in NSCLC cell lines. Suppressing the expression of ARK5 can increase sensitivity to cisplatin via a mechanism involving EMT. We suggest that ARK5 is a potential drug target for combating drug resistance and regulating EMT in NSCLC cells.

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## Disclosure of conflict of interest

None.

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