

LncRNA WT1-AS/miR-494-3p Regulates Cell Proliferation, Apoptosis, Migration and Invasion via PTEN/PI3K/AKT Signaling Pathway in Non-Small Cell Lung Cancer

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Background: Non-small cell lung cancer (NSCLC) is one of the most common malignancies with the highest morbidity and mortality worldwide. Long non-coding RNAs (lncRNAs) are recently recognized as noteworthy regulators of different tumors, counting NSCLC. However, the biological functions and regulatory mechanism of lncRNA WT1-AS in NSCLC progression still stay uninvestigated.

Methods: WT1-AS and miR-494-3p levels in NSCLC cell lines were detected by real-time quantitative polymerase chain reaction (RT-qPCR). In the current study, the regulatory effects of WT1-AS/miR-494-3p axis on cellular behaviors of NSCLC cell lines (A549 and NCI-H1975) were evaluated by a variety of methods. Cell counting kit-8 (CCK-8) and EDU assays were adopted to assess NSCLC cell proliferation. Tunnel staining and flow cytometry assay were applied to determine cell apoptosis and cell cycle distribution. Besides, cell migration and invasion abilities were analyzed by performing wound healing and transwell assays. Meanwhile, the levels of key proteins related to NSCLC cell apoptosis and PTEN/PI3K/AKT pathway were examined using Western blot assay. In addition, luciferase reporter assays were used to determine the interaction between WT1-AS and miR-494-3p or miR-494-3p and PTEN.

Results: Visibly downregulated WT1-AS in NSCLC cell lines was obtained from Broad Institute Cancer Cell Line Encyclopedia (CCLE) database and further verified by performing RT-qPCR. Besides, miR-494-3p was the downstream target gene of WT1-AS and obviously up-regulated miR-494-3p in NSCLC cell lines was confirmed. WT1-AS overexpression suppressed cell proliferation, migration and invasion abilities while enhanced cell apoptosis of A549 and NCI-H1975 cells. Furthermore, upregulation of miR-494-3p distinctly reversed these inhibitory effects of WT1-AS overexpression on the tumorigenesis and progression of NSCLC. In addition, WT1-AS promoted PTEN expression and thereby inhibited activation of PI3K/AKT pathway by sponging miR-494-3p.

Conclusion: To conclude, lncRNA WT1-AS impeded cell proliferation, migration, invasion but accelerated cell apoptosis via negatively regulating miR-494-3p to mediate PTEN/PI3K/AKT pathway in NSCLC.

Keywords: lncRNA WT1-AS, miR-494-3p, PTEN/PI3K/AKT, tumor progression, non-small cell lung cancer; NSCLC

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Introduction

As the common type of lung cancer, non-small cell lung cancer (NSCLC) is one of the most severe diseases threatening human health.¹ NSCLC possesses the

characteristics of high malignancy and aggressiveness, and the metastases of lymph nodes and different organs are prone to occur in advanced stages of NSCLC^{2,3}. Accompanied by the advancement of molecular biology in lung cancer, related driver genes and targeted therapy have become the focus of lung cancer research.⁴

Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is one of the most pivotal signaling pathways for cell survival, differentiation and apoptosis in the body.⁵ Recent studies have shown that the activation of PI3K/AKT signaling pathway can modulate proliferation, apoptosis, cycle arrest, migration and invasion of lung cancer cells.^{6,7} Phosphatase and tensin homologue deleted on chromosome ten (PTEN), a lipid phosphatase that functions as a tumor suppressor, has been widely affirmed to suppress tumor progression through PI3K/AKT signaling pathway.⁸

MicroRNAs (miRNAs) are endogenous non-coding small RNA molecules, which mediate the gene expression at the post-transcriptional level by binding to mRNAs, thus participating in the regulation of various biological processes.⁹ Recently, it has been reported that miRNAs exert critical roles in the tumorigenesis and progression of cancer. Among these miRNAs, miR-494-3p is overexpressed widely in plenty of human tumors including NSCLC.^{10,11} Besides, miR-494-3p overexpression is correlated with the development, improvement and prognosis of tumors.^{12,13} Moreover, miR-494-3p has been proved to activate PI3K/AKT pathway by targeting PTEN.¹⁴

Long non-coding RNAs (lncRNAs) are untranslated transcripts with more than 200 nucleotides.¹⁵ There is a mutual regulatory relationship between lncRNAs and miRNAs. As a competitive interaction between RNA (ceRNA) and miRNA, lncRNAs participate in the regulation of target gene expression.¹⁶ A study has elucidated that lncRNA WT1-AS is downregulated in NSCLC tissues, and decreased levels of lncRNA WT1-AS predict poor survival of NSCLC patients.¹⁷ However, more studies are urgently required to reveal the biological function and mechanism of lncRNA WT1-AS in NSCLC.

Taken together, the present study established lncRNA WT1-AS-overexpressing or lncRNA WT1-AS/miR-494-3p-overexpressing NSCLC cell lines. Subsequently, the proliferation, apoptosis, migration and invasion of these NSCLC cells were determined to investigate the regulating effects of lncRNA WT1-AS/miR-494-3p axis on NSCLC progression. In addition, the association among WT1-AS, miR-494-3p and PTEN/PI3K/AKT pathway in NSCLC

was further explored. The results from the present study will deepen the understanding of roles and underlying mechanism of WT1-AS/miR-494-3p axis in NSCLC.

Materials and Methods

Cell Culture and Transfection

Normal human lung bronchial epithelial cell line (16-HBE) and human NSCLC cell lines including (A549, NCI-H1975, SK-MES-1) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and human NSCLC cell line (PLA-801D) was obtained from Ningbo Mingzhou Biotechnology Co., Ltd. Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Gibco, MA, USA) supplemented with 10% FBS, 100U/mL penicillin, and 100mg/mL streptomycin (Gibco, MA, USA) in a 5% CO₂ humidified atmosphere at 37°C.

A549 or NCI-H1975 cells (80–90% confluence) were transfected with overexpression plasmid for lncRNA WT1-AS, miR-494-3p mimic or the corresponding negative control (NC) using the lipofectamine 2000 transfection kit (Invitrogen, CA, USA) according to the manufacturer's protocols.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, CA, USA). Following manufacturer's instructions, total RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Tokyo, Japan). Then, real-time quantitative polymerase chain reaction (RT-qPCR) were performed using SYBR Green methods (TaKaRa, Tokyo, Japan). The PCR conditions were 40 cycles at 95°C for 30 s, 95°C for 5 s and 60°C for 30 s, followed by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Relative expressions level of lncRNA WT1-AS was normalized to the endogenous control GAPDH and relative expression level of miR-494-3p was normalized to the endogenous control U6. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primers were as follows: WT1-AS-F: 5'-GCCTCTCTGTCTCTTCTTTGT-3' and WT1-AS-R: 5'-sGCTGTGAGTCCTGGTGCTTAG-3'; miR-494-3p-F, 5'-GATACTCGAAGGAGAGGTTGTC-3' and miR-494-3p-R, 5'-GAGGTTTCCCGTGTATGTTTCAT-3'; GAPDH-F, 5'-TCGACAGTCAGCCGCATCTTCTTT-3' and GAPDH-R, 5'-ACCAATCCGTTGACTCCGACCTT-3'; U6-F: 5'-CTCGCTTC

GGCAGCAC-3' and U6-R: 5'-AACGCTTCACGAATTT GCGT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Briefly, after the designed transfection, 5×10^3 A549 or NCI-H1975 cells were seeded into 96-well plates and incubated for 24, 48, and 72 h under standard conditions. After that, 10 μ L of CCK-8 assay solution (Beyotime, Shanghai, China) was added into each well and sustained for another 4 h. Absorbance of each well at 450 nm was recorded by Micro-plate Reader (Bio-Rad, CA, USA). Cell proliferation rate (%) = $(\text{cells number}_{(24h/48h/72h)} - \text{cells number}_{(0h)}) / \text{cells number}_{(0h)} \times 100\%$

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

Transfected cells were fixed with 4% paraformaldehyde and then washed with PBS. Next, cells were incubated with EdU working solution for 2 h and stained with DAPI at room temperature for 15 min. Cells were observed under a fluorescent microscopy (Olympus, Tokyo, Japan).

TdT-Mediated dUTP Nick-End Labeling (TUNEL) Staining

After transfection, cells were fixed with 4% paraformaldehyde for 30 min. Then, the cells were added with TUNEL reagent (Roche, Basel, Switzerland) at 37°C for 1 h. The nuclei were visualized with DAPI solution (Beyotime, Shanghai, China). After washing with PBS, the cells were detected using a florescent microscope (Olympus, Tokyo, Japan).

Western Blotting Analysis

Protein lysates were collected from A549 or NCI-H1975 cells using RIPA Buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktail. Protein concentrations were estimated using bicinchoninic acid protein assay kit (CWBI, Beijing, China) and proteins were denatured for 10min at 95°C. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with non-fat milk in Tris-buffered saline and Tween 20. After that, the membranes were incubated with primary antibodies against Bax (Abcam, ab32503, 1:5000), Bcl-2 (Abcam, ab32124, 1:1000), Cleaved caspase3 (Abcam, ab2302, 1:500),

Caspase3 (Abcam, ab32150, 1:1000), PTEN (Abcam, ab32199, 1:10,000), p-PI3K (Abcam, ab182651, 1:1000), p-AKT (Abcam, ab38449, 1:1000) and GAPDH (Abcam, ab9485, 1:2000) at 4 °C overnight. On the second day, the membranes were incubated with the corresponding IgG-HRP secondary antibody (Abcam, ab205718, 1:20,000) at room temperature for 1 h. Signals were developed using ECL (Sigma-Aldrich, MO, USA) and analyzed by Image J software (NIH, USA).

Cell Cycle Assay

Following transfection, the cells were collected with trypsin. Then, cells were washed once with 1×PBS and centrifuged for 5 min at 1500×g. Next, 70% pre-cooled ethanol was used to fix cells at 4°C overnight. After centrifugation, cells were mixed with propidium iodine (PI; 10 μ g/mL; Beyotime, Shanghai, China) on ice for 30 min. Then, the stained cells were subsequently analyzed by a flow cytometry (Bio-Rad, CA, USA).

Wound Healing Assay

Briefly, 5×10^5 cells were seeded in 6-well plates. After the cells were grown to 100% confluence, a wound was scratched using a 10- μ L pipette tip. Then, the cells were starved and cultured for 24 h. The scratch areas at 0 h and 24 h were photographed with a microscope (Leica, Wetzlar, Germany).

Transwell Invasion Assay

Cell invasion was determined by performing transwell assay. Briefly, the Matrigel (Sigma, MO, USA) was coated on the filter surfaces of the transwell chamber (Corning, MA, USA). Cell suspension (1×10^5) was prepared using serum-free medium and seeded in the upper chamber. Then, the lower chamber was filled with complete medium. After 24-h incubation, the cells penetrating the Matrigel were fixed with methanol and stained with 0.5% crystal violet. Cell numbers were counted by an inverted microscope (Leica, Wetzlar, Germany).

Luciferase Reporter Assay

Mutant (MUT) and wild-type (WT) sequences of lncRNA WT1-AS containing the 3'UTR were amplified and cloned into a pGL3-Basic reporter vector (Promega, WI, USA). 293-T cells from miR-NC and miR-494-3p groups were placed in 24-well plates and transfected with pGL3-WT1-AS-MUT and pGL3-WT1-AS-WT plasmids (Promega, WI, USA). After cultured for 48 h at 37°C, cells were

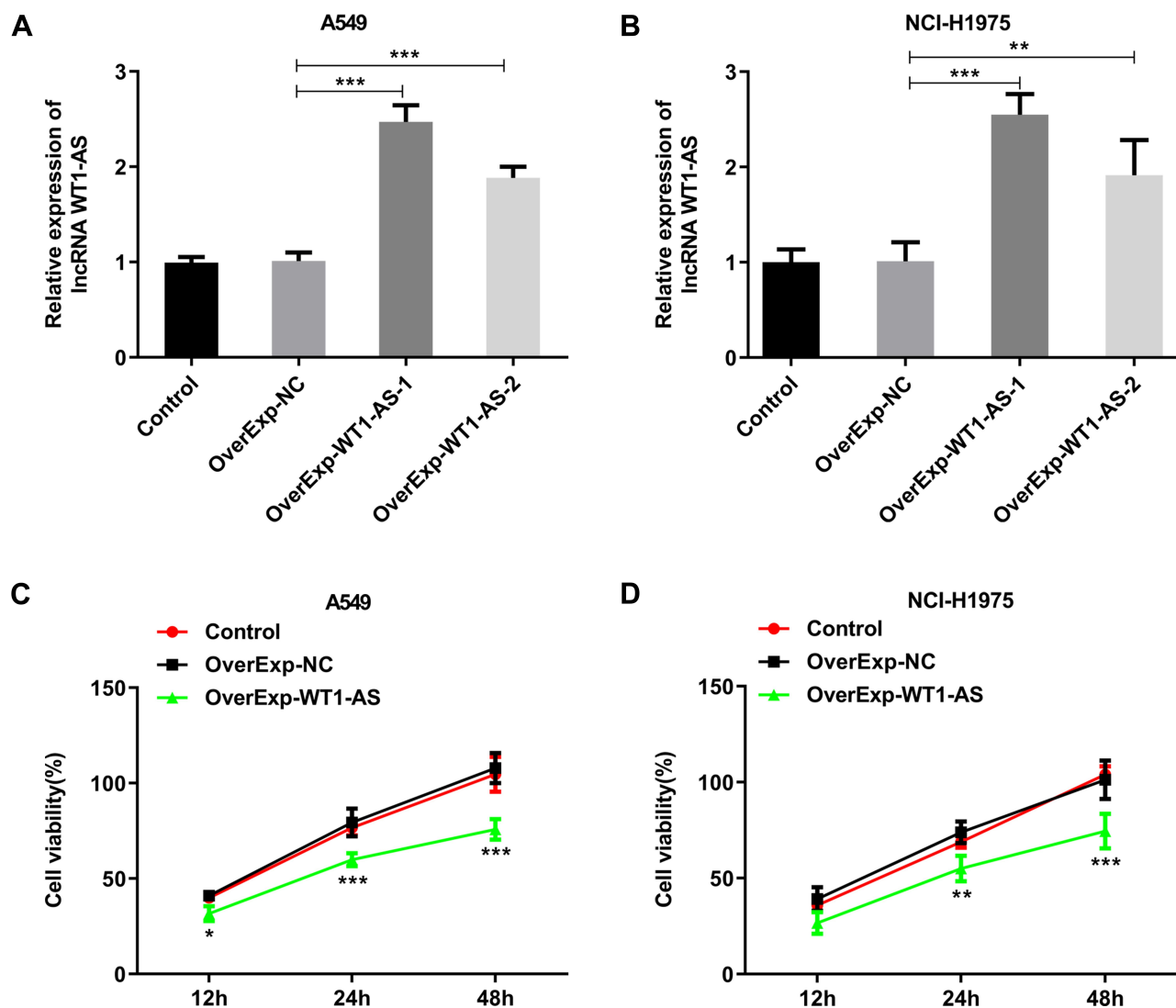


Figure 2 Overexpressed WT1-AS suppressed NSCLC cell proliferation. (A and B) RT-qPCR was adopted to validate the transfection efficiency in A549 cells and NCI-H1975 cells after transfection with WT1-AS overexpression vectors. (C and D) CCK-8 assay was performed to evaluate the effects of overexpressed WT1-AS on the proliferation of A549 and NCI-H1975 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Overexpressed WT1-AS Restrained NSCLC Cell Migration and Invasion Capacities

Subsequently, we evaluated the roles of WT1-AS in NSCLC cell metastasis by constructing WT1-AS overexpression cells. It was observed that upregulation of WT1-AS visibly reduced the migrative (Figure 7A and B) and invasive (Figure 7C and D) abilities of A549 cells. In addition, the migrative (Figure 7E and F) and invasive (Figure 7G and H) abilities of NCI-H1975 cells showed the similar tendency to be suppressed by WT1-AS overexpression. These results suggested that upregulation of WT1-AS restrained NSCLC metastasis in vitro.

WT1-AS Could Sponge miR-494-3p to Upregulate Its Target Gene PTEN, Thereby Suppressing PI3K/AKT Pathway in NSCLC

Furthermore, we speculated that WT1-AS functioned as a miRNA sponge in NSCLC. Based on the prediction from DIANA Tools, miR-494-3p was predicted to bind with WT1-AS (Figure 8A). Post 48 h transfection, transfection efficiency was verified by RT-qPCR, which validated that miR-494-3p level greatly increased in A549 cells following treatment of miR-494-3p mimic (Figure 8B). Luciferase reporter assay revealed that upregulation of miR-494-3p significantly decreased the luciferase activity

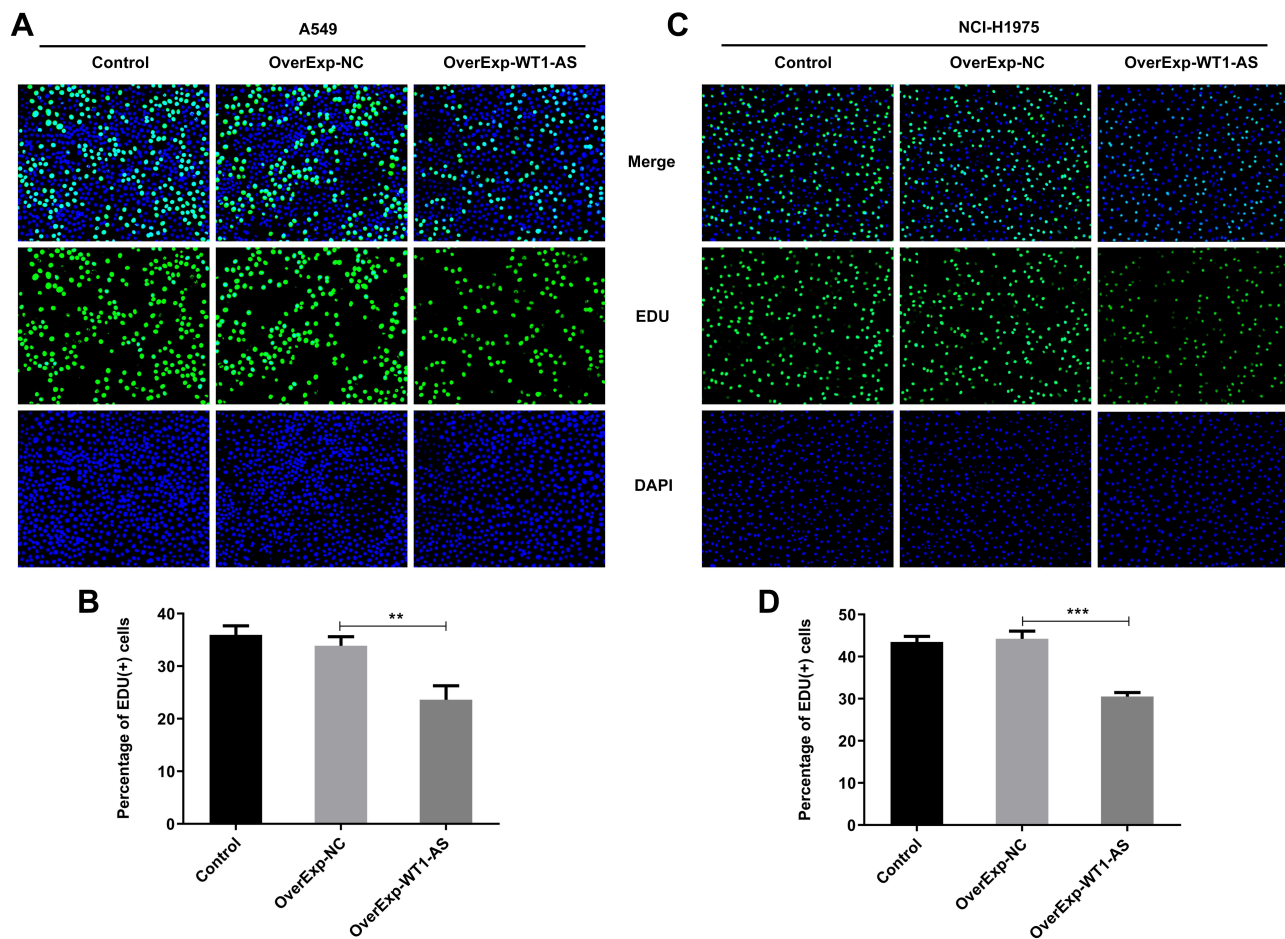


Figure 3 Overexpressed WT1-AS suppressed NSCLC cell proliferation. **(A and B)** EdU-positive cells of A549 were assessed by EdU staining to evaluate the effects of overexpressed WT1-AS on NSCLC cell proliferation. **(C and D)** EdU-positive cells of NCI-H1975 cells were assessed by EdU staining to evaluate the effects of overexpressed WT1-AS on NSCLC cell proliferation. ** $p < 0.01$, *** $p < 0.001$.

of WT1-AS-WT while had no inhibitory effect on the luciferase activity of WT1-AS-MUT (Figure 8C). As hypothesized, WT1-AS overexpression remarkably down-regulated miR-494-3p level (Figure 8D). These data together confirmed that WT1-AS sponged miR-494-3p to negatively regulate miR-494-3p expression. Besides, in comparison with that in 16-HBE cells, miR-494-3p expression was visibly enhanced in human NSCLC cell lines (A549, NCI-H1975, SK-MES-1, PLA-801D) (Figure 8E). In addition, the binding sites of miR-494-3p to PTEN were predicted based on bioinformatics website Starbase (Figure 8F). Overexpressed miR-494-3p suppressed the luciferase activity of PTEN-WT while had no obvious influence on that of PTEN-MUT (Figure 8G). Given that PTEN loss is the common abnormality in NSCLC, we further investigated whether PTEN expression was modulated by WT1-AS in NSCLC cell lines. Western blot analysis found a dramatic increase in PTEN, together

with decreases in its downstream targets (p-PI3K and p-AKT), following WT1-AS overexpression (Figure 8H). WT1-AS inactivated PI3K/AKT signaling pathway via upregulating PTEN. These findings suggested that WT1-AS-mediated function in NSCLC might be associated with PTEN and PI3K/AKT pathway.

miR-494-3p Counteracted WT1-AS Overexpression-Caused Anti-Proliferative and Pro-Apoptotic Effects

The above findings confirmed that elevation of WT1-AS exhibited significant anti-proliferative and pro-apoptotic effects in NSCLC cell lines. The decreased proliferation viability caused by WT1-AS overexpression was reversed by transfection of miR-494-3p mimic (Figure 9A). Furthermore, reduced TUNEL positive cells evidenced that overexpressed miR-494-3p obviously reserved the

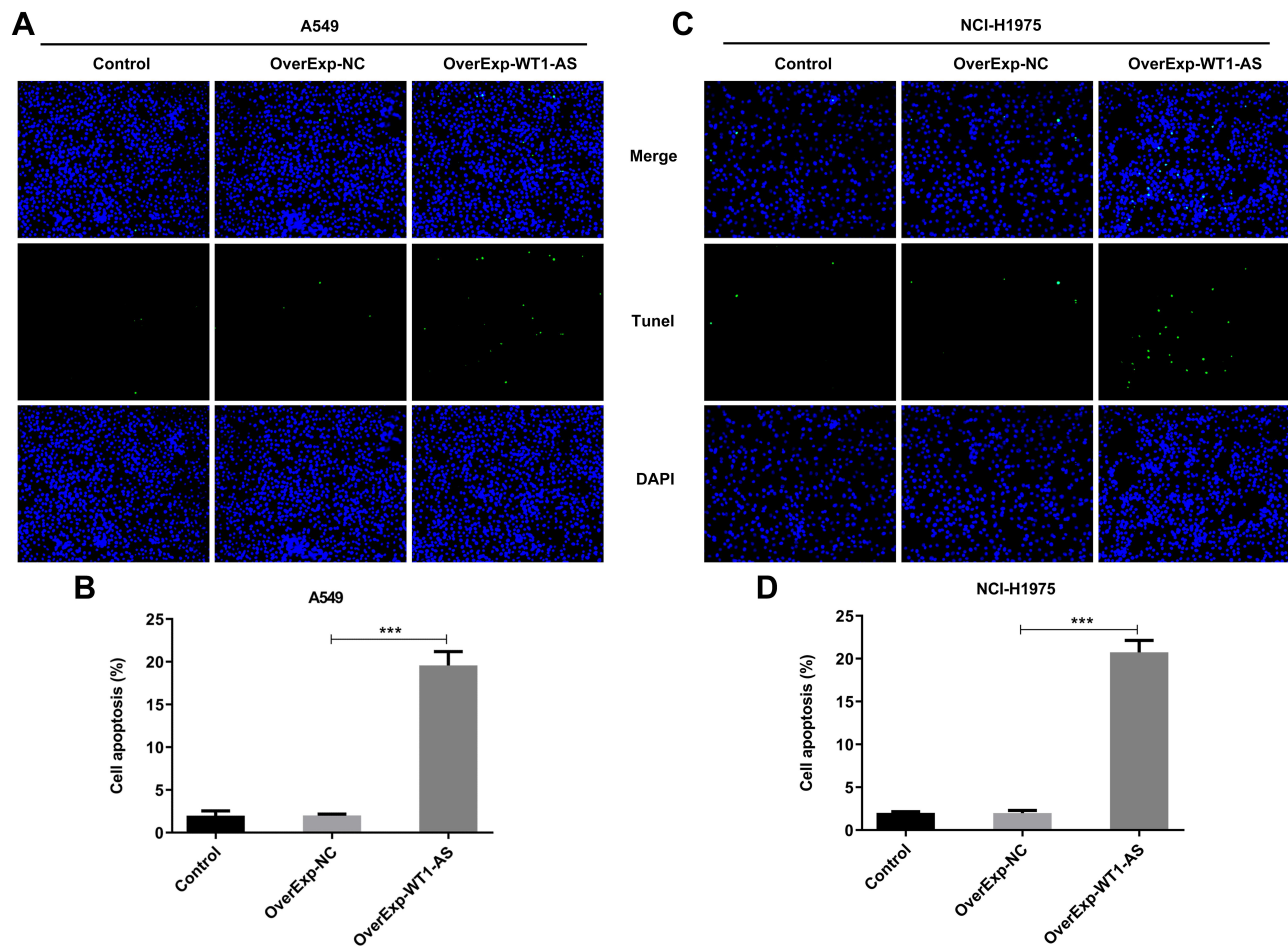


Figure 4 Overexpressed WT1-AS boosted NSCLC cell apoptosis. **(A)** TUNEL staining was applied to assess the effects of overexpressed WT1-AS on the apoptosis of A549 cells. **(B)** Percentage of TUNEL-positive A549 cells by TUNEL staining. **(C)** TUNEL staining was applied to assess the effects of overexpressed WT1-AS on the apoptosis of NCI-H1975 cells. **(D)** Percentage of TUNEL-positive NCI-H1975 cells by TUNEL staining. *** $p < 0.001$.

promoting effects of WT1-AS overexpression on NSCLC cell apoptosis (Figure 9B, C). Besides, Western blot assay for the detection of Bcl-2, Bax and cleaved caspase-3 levels further confirmed that the pro-apoptotic effects of WT1-AS overexpression in NSCLC were dramatically abolished by upregulation of miR-494-3p (Figure 9D).

miR-494-3p Counteracted WT1-AS Overexpression-Caused Anti-Migrative and Anti-Invasive Effects

As expected, the suppressing effects of WT1-AS elevation on cell migration were significantly restrained upon miR-494-3p promotion (Figure 10A and B). In addition, the decreased invasive ability induced by WT1-AS overexpression showed the similar tendency to be reversed by upregulation of miR-494-3p (Figure 10C and D). These results represented that miR-494-3p promotion abolished

the suppressing influence of WT1-AS overexpression on NSCLC cell metastasis in vitro.

WT1-AS Regulated PTEN/PI3K/AKT Signal Pathway via Modulating miR-494-3p in NSCLC Cells

miR-494-3p has been reported to promote PI3K/AKT pathway by targeting PTEN. To investigate whether WT1-AS affected PTEN/PI3K/AKT signal pathway via miR-494-3p, we then performed Western blotting analysis to explore the expression changes of PTEN, p-PI3K and p-AKT. Upregulated PTEN level and downregulated p-PI3K and p-AKT levels owing to WT1-AS elevation were rescued by miR-494-3p introduction (Figure 11). To sum up, the inhibitory effects of overexpressed WT1-AS on PI3K/AKT pathway was reversed by miR-494-3p promotion via targeting PTEN.

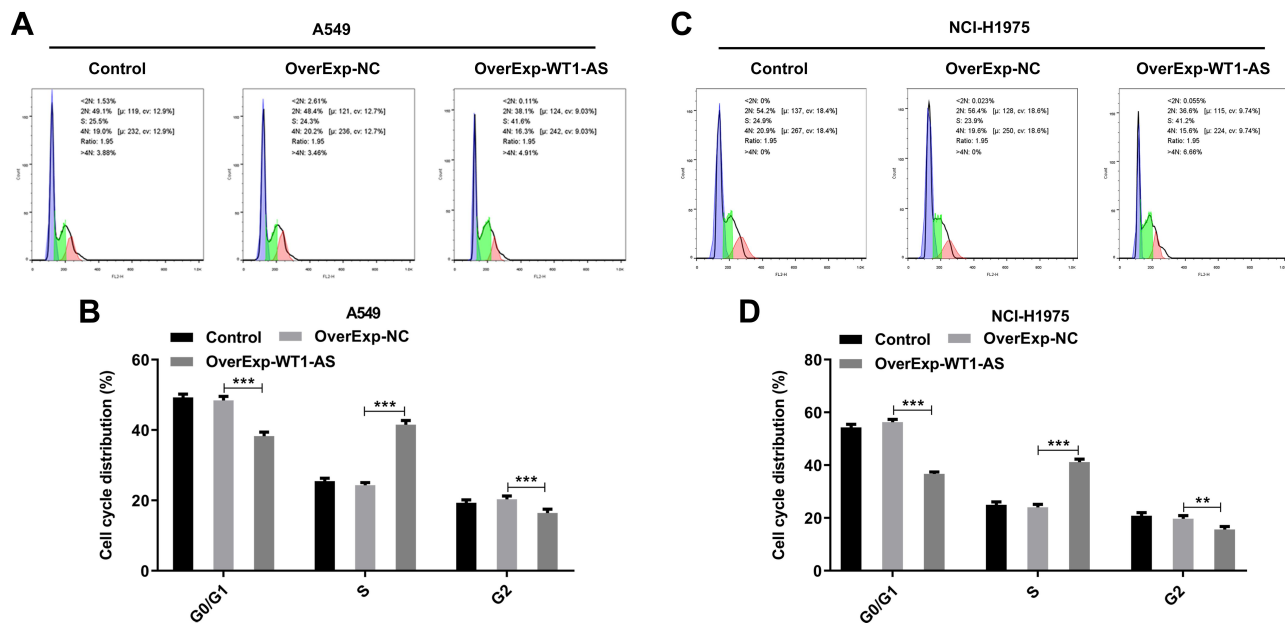


Figure 5 Overexpressed WT1-AS arrested cell cycle of NSCLC. **(A)** Flow cytometry analysis was performed for examining cell cycle of A549 cells. **(B)** Quantitative analysis of cell cycle distribution in A549 cells. **(C)** Flow cytometry analysis was performed for examining cell cycle of NCI-H1975 cells. **(D)** Quantitative analysis of cell cycle distribution in NCI-H1975 cells. ** $p < 0.01$, *** $p < 0.001$.

Discussion

Lung cancer is one disease with relatively high morbidity and mortality, of which NSCLC accounts for a large proportion.¹ Therefore, the study on the corresponding molecular mechanism responsible for proliferation and metastasis in NSCLC is very important for the early diagnosis and treatment. The abnormal expression of lncRNAs is closely associated with the tumorigenesis and progression of NSCLC, and lncRNAs may be potentially efficient biomarkers for early diagnosis and therapy of NSCLC.^{18,19} The primary results of the present study provide new insights into the modulatory role of WT1-AS/miR-494-3p in NSCLC progression via PTEN/PI3K/AKT pathway.

Considerable evidence has shown that WT1-AS is associated with the progression of several types of tumors through regulating cell proliferation and metastasis.^{20,21} Besides, WT1-AS is downregulated in NSCLC tissues and in negative correlation with poor survival of NSCLC patients.¹⁷ However, the functions of WT1-AS in NSCLC cells are still unclear. Here, we reported that WT1-AS was significantly downregulated in NSCLC cell lines. WT1-AS promotion inhibited the proliferation, migration, invasion abilities, but induced the apoptosis in NSCLC cells. These results pointed out WT1-AS as a tumor suppressor in NSCLC.

PI3Ks family, consisted in PI3K/AKT signaling pathway, are important oncology targets through regulation of

various cellular processes in research of antitumor drugs.²² The tumor suppressor PTEN, decreased or absent in many tumors, possesses a dual specific phosphatase activity.²³ PTEN can block the biological processes to inhibit the development of tumors through inactivating PI3K/AKT signaling pathway.⁸ It has also been documented that PTEN/PI3K/AKT signaling pathway is closely related to NSCLC cell proliferation, migration and invasion. This study verified that WT1-AS was a negative regulator of cell cycle and inactivated PTEN/PI3K/AKT pathway in NSCLC cells.

Based on the ceRNA hypothesis, lncRNAs regulate expression of target genes via absorbing miRNAs.¹⁶ A recent study has also revealed that WT1-AS levels are negatively correlated with miR-494-3p levels in glioma tissues and cell lines.²⁴ Consistently, our results also confirmed that miR-494-3p was a target of WT1-AS, and it was negatively regulated by WT1-AS in NSCLC cells. Importantly, Favarsani et al have reported that miR-494-3p is a tumor driver for lung cancer, and it could improve the growth and metastasis capabilities of NSCLC cells.¹⁰ Besides, Zhu et al have revealed the promoting impact of miR-494-3p on the proliferation and metastasis in endometrial cancer cells by regulating PTEN/PI3K/AKT pathway.²⁵ In this study, it was experimented that miR-494-3p reversed the anti-cancer functions mediated by WT1-AS overexpression in NSCLC cells.

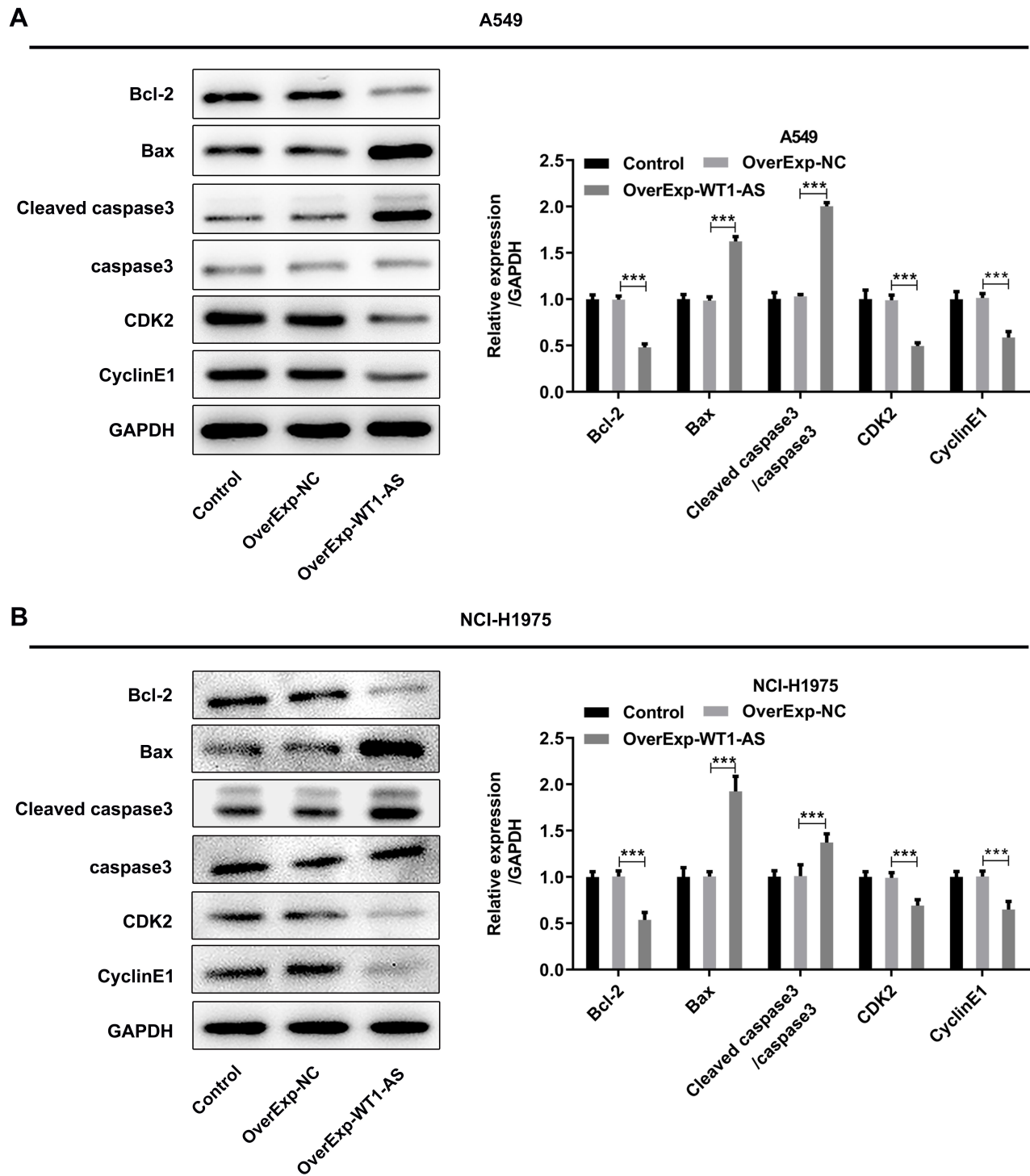


Figure 6 Levels of key proteins related to cell apoptosis and cell cycle. (A and B) Western blotting analysis was applied to determine expression levels of Bcl-2, Bax, cleaved caspase-3, CDK2 and CyclinE1. *** $p < 0.001$.

Taken together, our study demonstrated that WT1-AS performed anti-cancer effects in NSCLC by suppressing miR-494-3p. WT1-AS decoyed miR-494-3p to upregulate its target gene PTEN, thereby inactivating PI3K/

AKT pathway in NSCLC. Hence, WT1-AS may be a possible therapeutic target for NSCLC and this study might provide new potential therapeutic strategy for NSCLC.

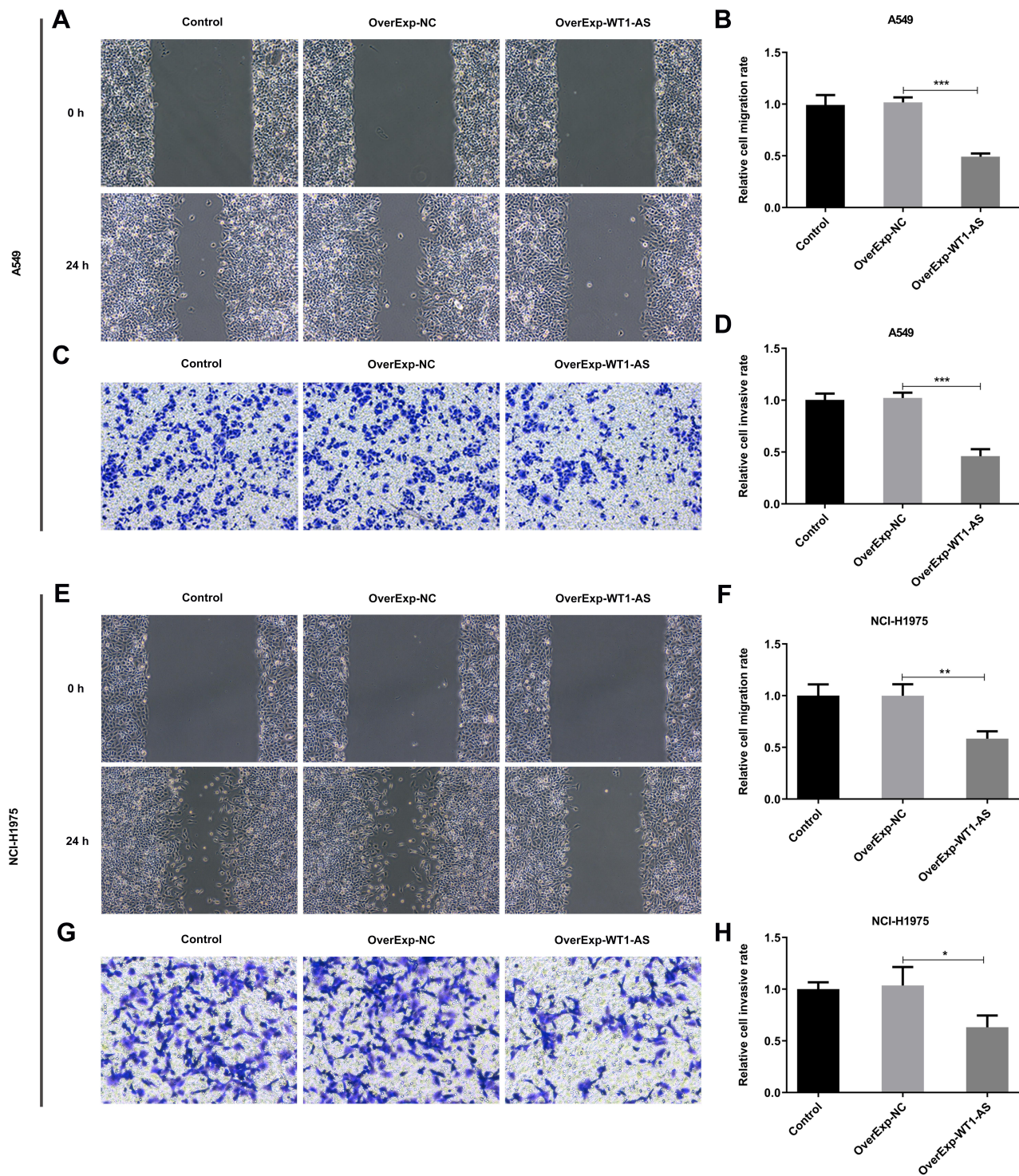


Figure 7 Overexpressed WT1-AS inhibited the migrative and invasive abilities of NSCLC cells. **(A)** Wound healing assay was conducted to evaluate the effects of overexpressed WT1-AS on the migrative ability of A549 cells. **(B)** Quantitative analysis of cell migration in A549 cells. **(C)** Transwell assay was conducted to evaluate the effects of overexpressed WT1-AS on the invasive ability of A549 cells. **(D)** Quantitative analysis of cell invasion in A549 cells. **(E)** Wound healing assay was conducted to evaluate the effects of overexpressed WT1-AS on the migrative ability of NCI-H1975 cells. **(F)** Quantitative analysis of cell migration in NCI-H1975 cells. **(G)** Transwell assay was conducted to evaluate the effects of overexpressed WT1-AS on the invasive ability of NCI-H1975 cells. **(H)** Quantitative analysis of cell invasion in NCI-H1975 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

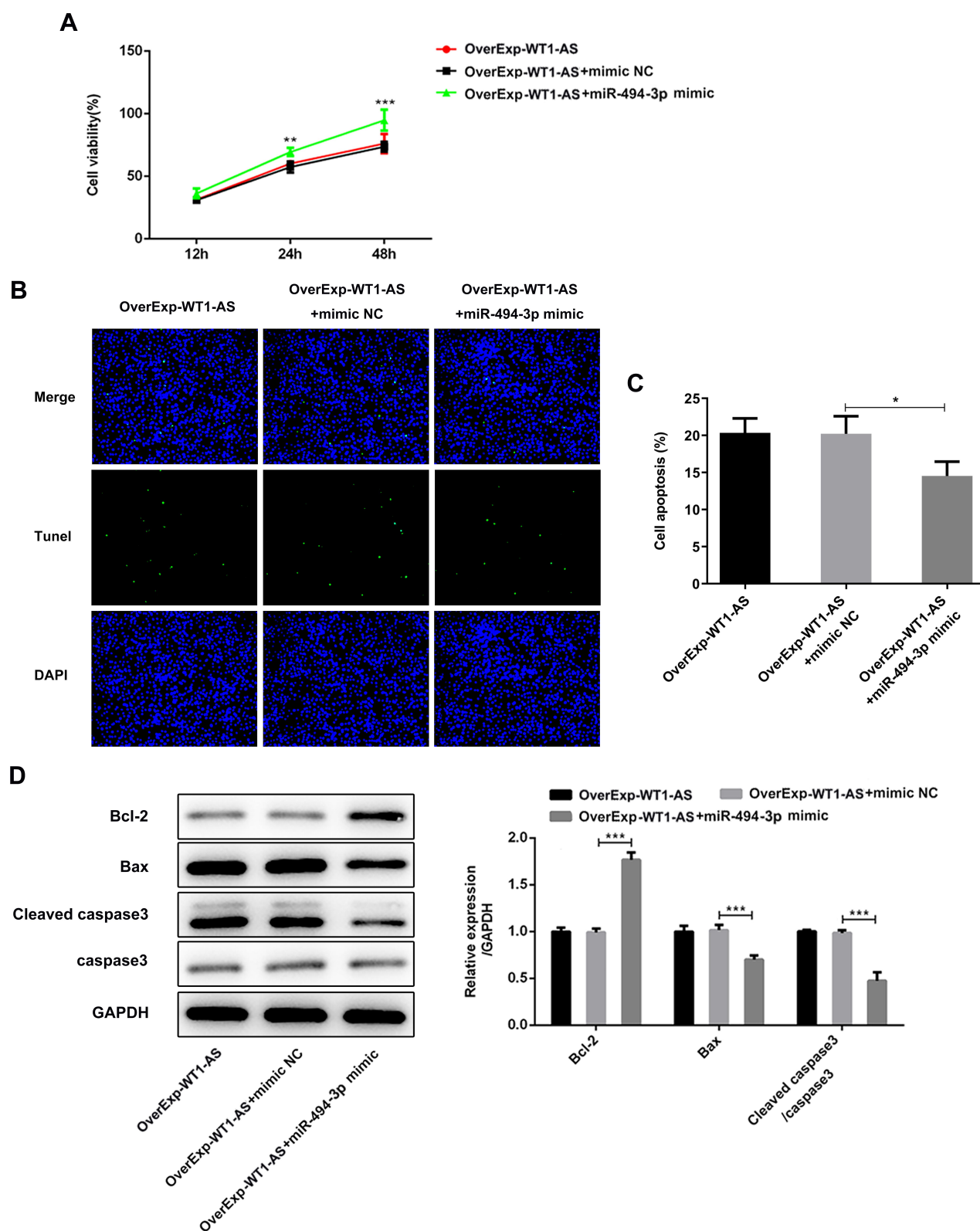


Figure 9 miR-494-3p counteracted the anti-proliferative and pro-apoptotic effects of overexpressed WT1-AS. **(A)** CCK-8 assay was performed to evaluate the influence of miR-494-3p promotion on the anti-proliferative effects of overexpressed WT1-AS in A549 cells. **(B and C)** TUNEL staining was applied to assess the influence of miR-494-3p promotion on the pro-apoptotic effects of overexpressed WT1-AS in A549 cells. **(D)** Western blot assay was conducted for the detection of anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Bax and cleaved caspase-3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

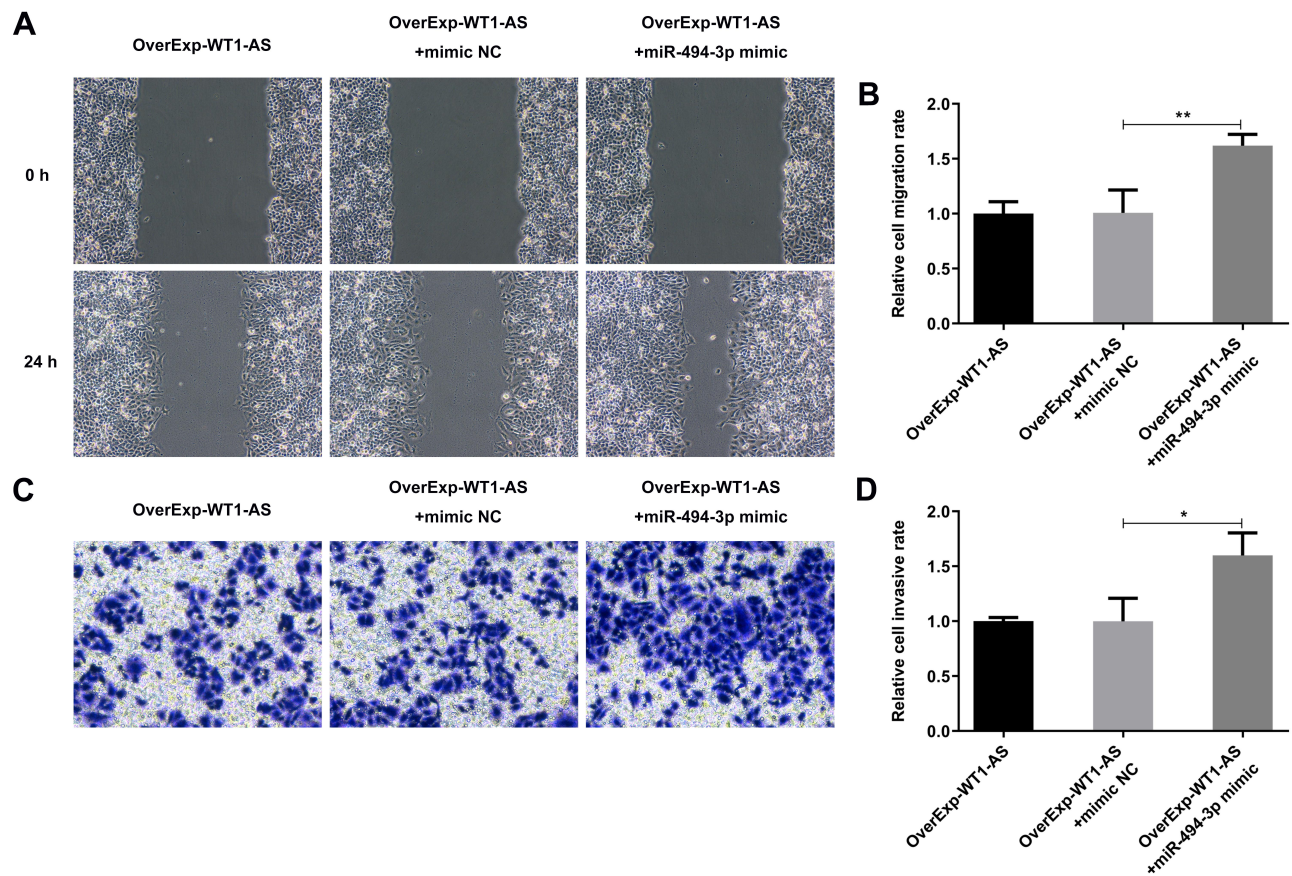


Figure 10 miR-494-3p counteracted the suppressing effects of overexpressed WT1-AS on cell migration and invasion. (A) Wound healing assay was conducted to evaluate the influence of miR-494-3p promotion on the anti-migrative effects of overexpressed WT1-AS in A549 cells. (B) Quantitative analysis of cell migration in A549 cells. (C) Transwell assay was conducted to evaluate the influence of miR-494-3p promotion on the anti-invasive effects of overexpressed WT1-AS in A549 cells. (D) Quantitative analysis of cell invasion in A549 cells. * $p < 0.05$, ** $p < 0.01$.

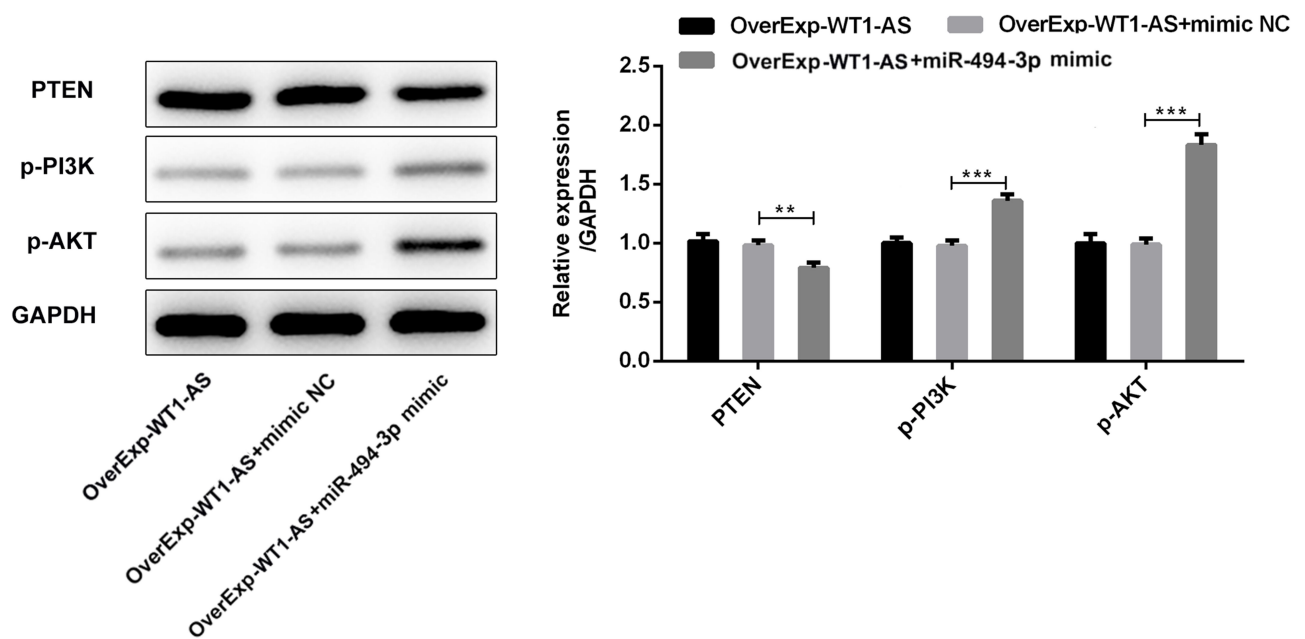


Figure 11 The suppressing effects of WT1-AS promotion on PI3K/AKT pathway was reversed by overexpressed miR-494-3p via targeting PTEN. Representative bands and quantitative analysis of PTEN/PI3K/AKT by performing Western blotting analysis. ** $p < 0.01$, *** $p < 0.001$.

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Disclosure

The authors declare that they have no conflicts of interest.

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