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Silencing the *FOLR2* Gene Inhibits Cell Proliferation and Increases Apoptosis in the NCI-H1650 Non-Small Cell Lung Cancer Cell Line via Inhibition of AKT/Mammalian Target of Rapamycin (mTOR)/Ribosomal Protein S6 Kinase 1 (S6K1) Signaling

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Background: The *FOLR2* gene encodes folate receptor-beta (FR-beta), which is expressed by tumor-associated macrophages. The effects of *FOLR2* gene expression in non-small cell lung cancer (NSCLC) remains unknown. This study aimed to investigate the effects of *FOLR2* gene expression and gene silencing in human NSCLC cell lines and normal human bronchial epithelial (HBE) cells *in vitro*.

Material/Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to detect the expression of the *FOLR2* gene, cell cycle and apoptosis-associated genes in normal HBE cells and the NSCLC cell lines, A549, NCI-H1299, NCI-H1650, and NCI-H460. Using small interfering RNA (siRNA), or silencing RNA, *FOLR2* gene silencing was performed for NCI-H1650 cells. Cell counting kit-8 (CCK-8) was used to measure cell viability. Cell cycle and apoptosis were determined using flow cytometry. Western blot evaluated the expression of Akt, mTOR, and S6K1 signaling.

Results: Expression of the *FOLR2* gene was increased in NSCLC cells compared with normal HBE cells. Silencing of the expression of the *FOLR2* gene in NCI-H1650 cells reduced cell viability, increased cell apoptosis, and arrested cells in the G1 phase of the cell cycle, decreased the expression of cyclin D1, upregulated expression of cell cycle inhibitors, p21 and p27, upregulated the expression of Bax/Bcl-2, and inhibited phosphorylation of AKT, mTOR, and S6K1.

Conclusions: Silencing of the *FOLR2* gene inhibited phosphorylation of AKT, mTOR, and S6K1, inhibited cell proliferation and increased apoptosis in the NCI-H1650 human NSCLC cell line.

MeSH Keywords: **Apoptosis • Carcinoma, Non-Small-Cell Lung • Cell Cycle • Folate Receptor 2**

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Background

In the United States (US), lung cancer is the most common type of malignancy, with almost 222,500 new cases diagnosed in 2017, resulting in 155,870 deaths within one year [1]. Worldwide, lung cancer remains a large health burden and cause of mortality. The two main histopathological types of lung cancer include the highly aggressive small cell lung cancer (SCLC), which has a high rate of recurrence [2]. Non small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of cases, and can be divided histologically into three types of NSCLC, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [3].

The European Prospective Investigation into Cancer and Nutrition (EPIC) study reported that folic acid deficiency was associated with an increased risk of lung cancer [4]. Folates are single-carbon carriers that have a role in the metabolism of purine and some amino acids involved in internal cell transport mechanisms [5]. Folic acid required for cell growth and cell proliferation is not manufactured by mammalian cells and is usually obtained from the diet or dietary supplements. The main function of the folate receptor is to introduce extracellular folic acid and folic acid derivatives into cells through endocytosis. The folate receptor is a glycoprotein consisting of four subtypes of α , β , γ and δ , in which FR- α and FR- β are anchored on the cell membrane through glycosylphosphatidylinositol (GPI) [6,7].

The folate receptor gene, *FOLR*, is located on chromosome 11 (q13.3-114.1). The genes *FOLR1*, *FOLR 2*, *FOLR 3* and *FOLR 4* encode the proteins FR- α , β , γ , and δ , respectively. The protein FR- γ is a secretory protein and FR- δ is a T-cell regulatory protein [8,9]. FR- α has been extensively studied and has been shown to play an important role in the diagnosis and treatment of tumors [10,11]. Also, FR- α is widely expressed in several tissues, including the kidney, breast, lung, and placenta [12,13]. The amino acid sequence for FR- α has 68% and 71% homology with FR- β and FR- γ , respectively. Although closely related in terms of its amino acid sequence, FR- β , encoded by the *FOLR2* gene, has a different tissue distribution and cellular specificity and is associated with pro-inflammatory mononuclear phagocytes [14]. The *FOLR2* gene has been shown to be expressed by malignant cells, including myelogenous leukemia cells, but has also been demonstrated to be mainly expressed by tumor-associated macrophages (TAMs) [15–17]. To our knowledge, no previous studies have been undertaken to investigate the effects of the expression of the *FOLR2* gene, or its lack of expression, in human NSCLC cells.

Several molecular signaling pathways are now recognized to be involved in cell survival in human NSCLC, including the c-Jun N-terminal kinase (JNK) signaling pathway, the matrix

metalloproteinase-2 (MMP-2) signaling pathway, the B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (BAX) signaling pathway, the phosphatidylinositol 3-kinase (PI3K), AKT, nuclear factor (NF)- κ B signaling pathway, and the solute carrier family 10 member 2 (SLC10A2), peroxisome proliferator-activated receptor gamma (PPAR γ), phosphatase and tensin homolog (PTEN), mechanistic target of rapamycin (mTOR) signaling pathway [18–21]. Previously published studies have shown that activation of the AKT, mTOR, and mTOR substrate S6 kinase 1 (S6K1) signaling pathway can contribute to tumorigenesis, metastasis, and angiogenesis in several types of malignant tumors [22–25]. However, the AKT/mTOR/S6K1 signaling pathway in human NSCLC remains poorly understood.

Therefore, this study aimed to investigate the effects of *FOLR2* gene expression and gene silencing on cell proliferation, the cell cycle, and apoptosis in human NSCLC cell lines and normal human bronchial epithelial (HBE) cells *in vitro*, including the components of the AKT/mTOR/S6K1 signaling pathway.

Material and Methods

Cell culture

Normal human bronchial epithelial cells (HBEs) and human non-small cell lung cancer (NSCLC) cell lines, A549, NCI-H1299, NCI-H1650, and NCI-H460, were obtained from Invitrogen (San Mateo, CA, USA). Cells were cultured in RPMI 1640 medium containing 20% fetal bovine serum (FBS) and incubated at 37°C and 5% CO₂. When cell confluence reached 80%, cells were digested with 0.25% trypsin and sub-cultured.

Cell transfection with small interfering RNA (siRNA), or silencing RNA, and grouping

The siRNA, or silencing RNA, for *FOLR2* (si-*FOLR2*) and empty control plasmids were purchased from RiboBio (Guangzhou, China). NCI-H1650 cells were seeded in 6-well plates (1.0×10⁵) for 24 hours before transfection and divided into the following three groups: the control group (0.1% PBS), the NC group (treated with empty vector), and the si-*FOLR2* group (transfected with the small interfering RNA or siRNA plasmid).

Transient transfection was performed by Lipofectamine 2000 (Invitrogen, SanMateo, CA, USA) according to the manufacturer's protocol. A total of 20 μ M siRNA, control, NC, and 5 μ L Lipofectamine 2000 was added to Opti-MEM[®] reduced serum medium and incubated at 25°C for 10 min. Lipofectamine 2000 was mixed into each group and cultured in Opti-MEM[®] RPMI 1640 medium. After 6 hours in culture, the fluid was changed back to RPMI 1640 medium containing 10% FBS.

Cell viability assessed using cell counting kit-8 (CCK-8)

After transfection, NCI-H1650 cells were digested with 0.25% trypsin for 12, 24, and 48 hours. Cells were plated into 96-well plates at a seeding density of 1×10^4 cells per well and divided into three groups: the control group; the NC group; and the si-*FOLR2* group. Then, 10 μ L CCK-8 solution was added to cells for an additional 2 hours at 37°C. The optical density (OD) was measured at a wavelength of 450 nm (Thermo Fisher, MA, USA).

Flow cytometry

NCI-H1650 cells were digested with 0.25% trypsin and collected in 1.5 ml Eppendorf tubes and centrifuged at 3,500 rpm for 5 min. The apoptosis assay included washing the cells twice using washing buffer, and the suspension was cultured with an Annexin V-PE apoptosis kit and propidium iodide (PI) (Lianshu, Shanghai, China) in the dark at 25°C for 20min. Binding buffer was added to each well. Flow cytometry analyzed the cell samples within one hour.

Cell cycle was also studied using flow cytometry. Cells washed twice in PBS and fixed in ethanol at 4°C for 30 min, followed by centrifuging at 1,000 rpm for 5 min. Cells were washed and resuspended in PBS with RNase and PI (Lianshu, Shanghai, China) at 37°C for 30 min.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure *FOLR2* mRNA, cell cycle-related mRNA (for cyclin D1, p21, and p27), apoptosis-related mRNA (for BAX and Bcl-2) in the different cell groups. Total RNA was extracted from cultured cells with Trizol (Invitrogen, SanMateo, CA, USA) according to manufacturer's instructions. Total RNA was reverse transcribed using the OrimeScript™ RT reagent kit (TaKaRa, Otsu, Shiga, Japan), performed at 42°C for 1 h and at 70°C for 10 min. cDNA was used for amplification by SYBR Fast qPCR Mix (Invitrogen, SanMateo, CA, USA). The PCR reaction consisted of the following cycles: an initial cycle at 95°C for 5 min; followed by 30 cycles at 94°C for another 30 seconds; then at 55°C for 30 seconds; and at 70°C for 45 seconds. The primer sequences are summarized in Table 1. Amplified products were electrophoresed in 1.2% agarose gels. The amount of RNA was calculated using the $2^{-\Delta\Delta CT}$ method, and β -actin was used as an internal control [26].

Western blot

Total protein was extracted from cultured cells using lysis buffer and RIPA buffer added to each well, followed by centrifuging at 12,000 \times g for 10 min. The bicinchoninic acid (BCA) assay

Table 1. Primers used in qRT-PCR.

Gene	Primer	Sequence
FOLR2	Forward	5'-GCUGCUUCUGGUCUGUGUAGC-3'
	Reverse	5'-UACACAGACCAGAAGCAGCAG-3'
Cyclin D1	Forward	5'-GCGGAATTCTGTGCTGCGAAGTGAAAA-3'
	Reverse	5'-GCGAAGCTTTCAGATGTCACGTCCTCCG-3'
P21	Forward	5'-CGATGCCAACCTCCTCAACGA-3'
	Reverse	5'-TCGCAGACCTCCAGCATCCA-3'
P27	Forward	5'-TGCAACCGACGATTCTTACTCAA-3'
	Reverse	5'-CAAGCAGTGATGATCTGATAAACAAGGA-3'
Bax	Forward	5'-GAGCGAGTGTCTCCGGCGAATT-3'
	Reverse	5'-GCCACAAAGATGGTCACTGTCTGC-3'
Bcl-2	Forward	5'-TGGGATGCCTTTGTGGAAGTAT-3'
	Reverse	5'-GCTGATTTGACCATTTGCCTGA-3'
β -actin	Forward	5'-CATCCTGCGTCTGGACCT-3'
	Reverse	5'-TCAGGAGGAGCAATGATCTTG-3'

(Thermo Fisher, MA, USA) was used to quantify the proteins. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% gel was used to load the proteins, which were then transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked in 5% dried milk powder, Tris-buffered saline (TBS) and Tween-20.

Proteins were detected by incubation of the gels with different primary antibodies, including: rabbit anti-Bcl-2 (1: 1,000) (ab32124; Abcam, Cambridge, MA, USA), anti-BAX (1: 1500) (ab32503; Abcam, Cambridge, MA, USA); anti-FOLR2 (1: 1000) (ab56067; Abcam, Cambridge, MA, USA), anti-cyclin D1 (1: 1000) (ab134175; Abcam, Cambridge, MA, USA), anti-p21 (1: 1000) (ab109520; Abcam, Cambridge, MA, USA), anti-p27 (1: 5000) (ab32034; Abcam, Cambridge, MA, USA), anti- β -actin (1: 1000) (ab8227; Abcam, Cambridge, MA, USA), anti-AKT (1: 1000) (SAB4500797; Sigma, USA), anti-p-AKT (1: 1000) (SAB4301414; Sigma, USA), anti-mTOR (1: 2000) (ab2732, 1: 2000, Abcam, USA), anti-p-mTOR (S2448, ab84400, 1: 2000, Abcam, USA), anti-S6K1 (ab32529; Abcam, Cambridge, MA, USA), anti-p-S6K1 (S424) (1: 1000) (ab131436; Abcam, Cambridge, MA, USA). Following incubation with the primary antibody, the gels were washed with TBST, and then incubated with horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Proteintech, Chicago, IL, USA) as secondary antibodies. The blot was visualized using an enhanced chemiluminescence (ECL) system (GE Healthcare, Chicago, IL, USA) to detect the bands. The density of the blots was measured using the Quantity One software version 2.4 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

GraphPad Prism version 6.0 software was used for statistical analysis. All data were presented as the mean \pm standard

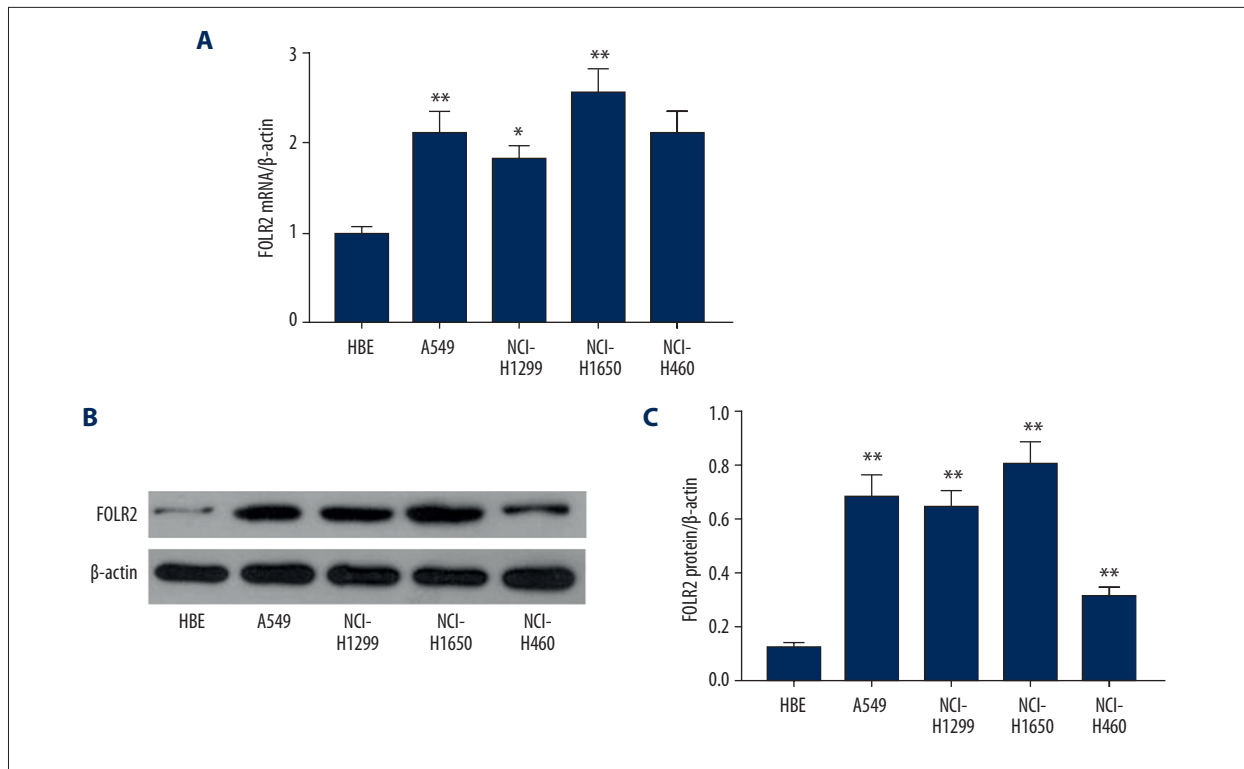


Figure 1. The mRNA and protein expression of *FOLR2* in normal human bronchial epithelial (HBE) cells and human non-small cell lung carcinoma (NSCLC) cell lines. **(A)** mRNA levels of *FOLR2* in normal human bronchial epithelial cells (HBEs) and human non-small cell lung carcinoma (NSCLC) cell lines, A549, NCI-H1299, NCI-H1650, and NCI-H460 detected using quantitative real-time polymerase chain reaction (qRT-PCR). **(B)** Protein levels of *FOLR2* in normal HBEs and NSCLC cell lines detected using Western blot. **(C)** Protein expression levels shown as bar diagrams. β -actin served as an internal control. Data are expressed as the mean \pm SD from three independent experiments. (* Compared with normal bronchial epithelial cells, * $P < 0.05$; ** $P < 0.01$).

deviation (SD). Differences were compared using one-way analysis of variance (ANOVA) following Tukey's range test for multiple comparisons. A p -value < 0.05 was considered to be statistically significant.

Results

Expression of *FOLR2* mRNA and *FOLR2* protein in normal human bronchial epithelial (HBE) cells and human non-small-cell lung cancer (NSCLC) cell lines

Normal human bronchial epithelial (HBE) cells and human non-small-cell lung cancer (NSCLC) cell lines were studied for the expression of *FOLR2* mRNA and protein levels. Almost all the NSCLC cell lines showed increased expression of *FOLR2* mRNA compared with the normal HBE cells (Figure 1A). A549 ($P < 0.01$), NCI-H1299 ($P < 0.05$) and NCI-H1650 ($P < 0.01$) showed the most significant increase in expression of *FOLR2* mRNA; the NCI-H460 cells showed expression levels of *FOLR2* mRNA that was comparable with the HBE cells.

The *FOLR2* protein levels of four NSCLC cell lines, A549, NCI-H1299, NCI-H1650, and NCI-H460, were significantly increased when compared with normal HBE cells ($P < 0.01$) (Figure 1B, 1C). However, because the NCI-H1650 human NSCLC cell lines showed the highest expression of *FOLR2* mRNA and *FOLR2* protein, this cell line was used for the further experiments, including silencing studies for *FOLR2*.

Expression of *FOLR2* mRNA and *FOLR2* protein following transfection with small interfering RNA (siRNA) (si*FOLR2*) in the human NSCLC cell line, NCI-H1650

To determine the transfection efficiency of *FOLR2* in human NSCLC NCI-H1650 cells, both mRNA and protein analysis were performed. Control and NC cells showed no significant difference in mRNA level. The si*FOLR2* group showed significantly reduced expression of *FOLR2* mRNA and *FOLR2* protein compared with the control and NC group ($P < 0.01$) (Figure 2A, 2B).

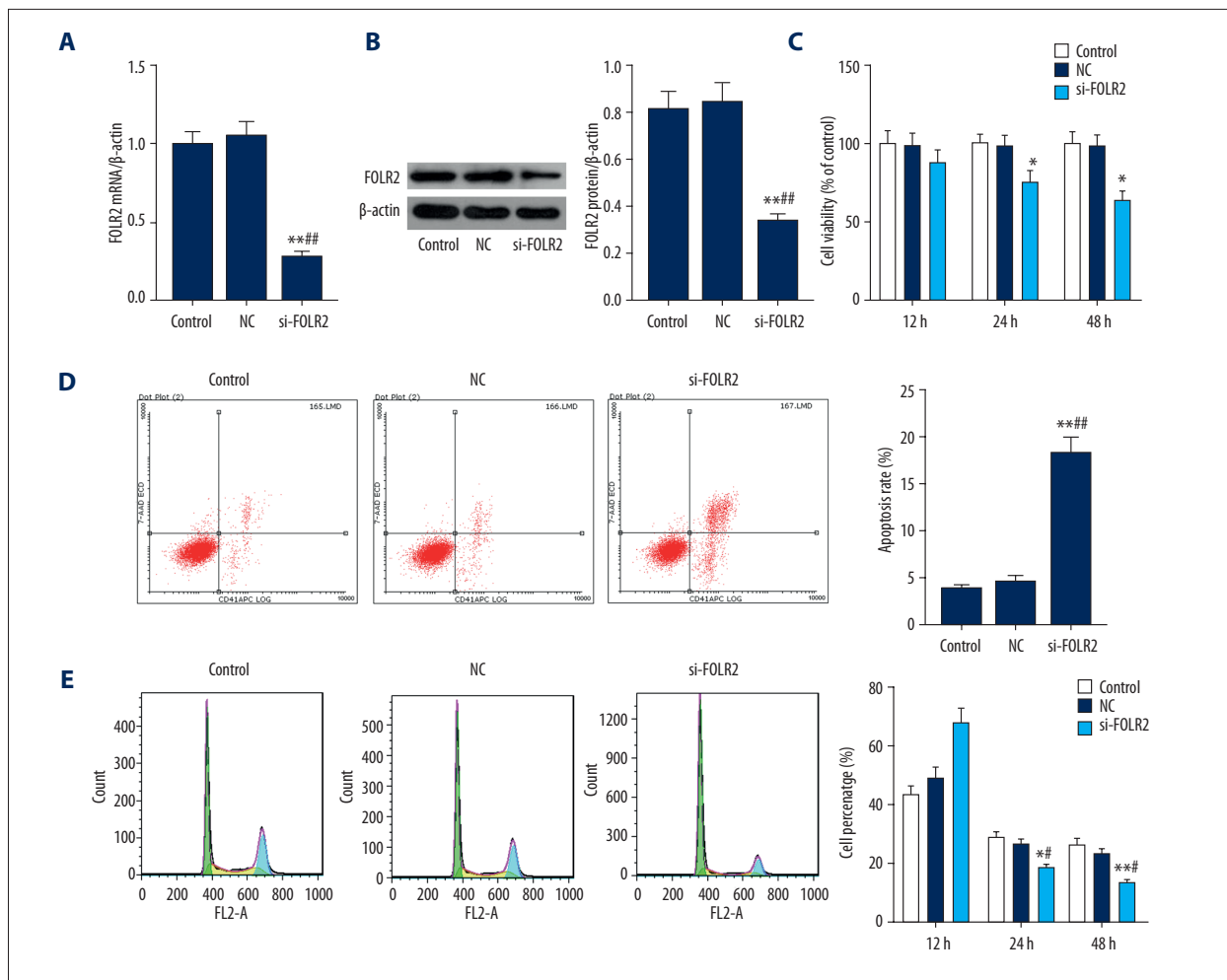


Figure 2. The effects of small interfering RNA (siRNA) silencing of *FOLR2* expression on cell proliferation, the cell cycle, and apoptosis of the NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells. **(A)** Following transfection with small interfering RNA (siRNA), or silencing RNA, mRNA expression of *FOLR2* was assessed in the NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells using quantitative real-time polymerase chain reaction (qRT-PCR). **(B)** Following transfection with siRNA, protein expression of *FOLR2* was detected in the NCI-H1650 human NSCLC cells using Western blot. **(C)** The effects of siRNA silencing of expression of the *FOLR2* gene reduced cell viability in the NCI-H1650 human NSCLC cells, assessed using cell counting kit-8. **(D)** The effects of siRNA silencing of expression of the *FOLR2* gene induced cell apoptosis in the NCI-H1650 human NSCLC cells, assessed using flow cytometry. **(E)** The effects of siRNA silencing of expression of the *FOLR2* gene affected the cell cycle in the NCI-H1650 human NSCLC cells, assessed using flow cytometry. β-actin served as an internal control. Data are expressed as the mean ±SD from three independent experiments. (* Compared with control, # compared with NC, **/### P<0.05; **/### P<0.01).

Silencing of *FOLR2* expression (si*FOLR2*) reduced the cell viability of the human NSCLC cell line, NCI-H1650

To investigate the cell viability of si*FOLR2* expression in NCI-H1650 cells, the cell counting kit-8 (CCK-8) assay was performed. As shown in Figure 2C, after 12 hours, the cell viability of the si*FOLR2* group had a slight but a non-significant difference when compared with the control or NC groups. After 24 h or 48 h in culture, the si*FOLR2* cell viability was significantly decreased compared to the control NCI-H1650 cells (P<0.05) (Figure 2C).

Silencing of *FOLR2* expression (si*FOLR2*) increased cell apoptosis of the human NSCLC cell line, NCI-H1650

The effect of *FOLR2* silencing on apoptosis of NCI-H1650 cells was detected by flow cytometry. The rate of cell apoptosis of the si*FOLR2* group was significantly increased when compared with the control or NC cells (P<0.01) (Figure 2D). This result indicated that *FOLR2* gene silencing could promote NCI-H1650 cell apoptosis.

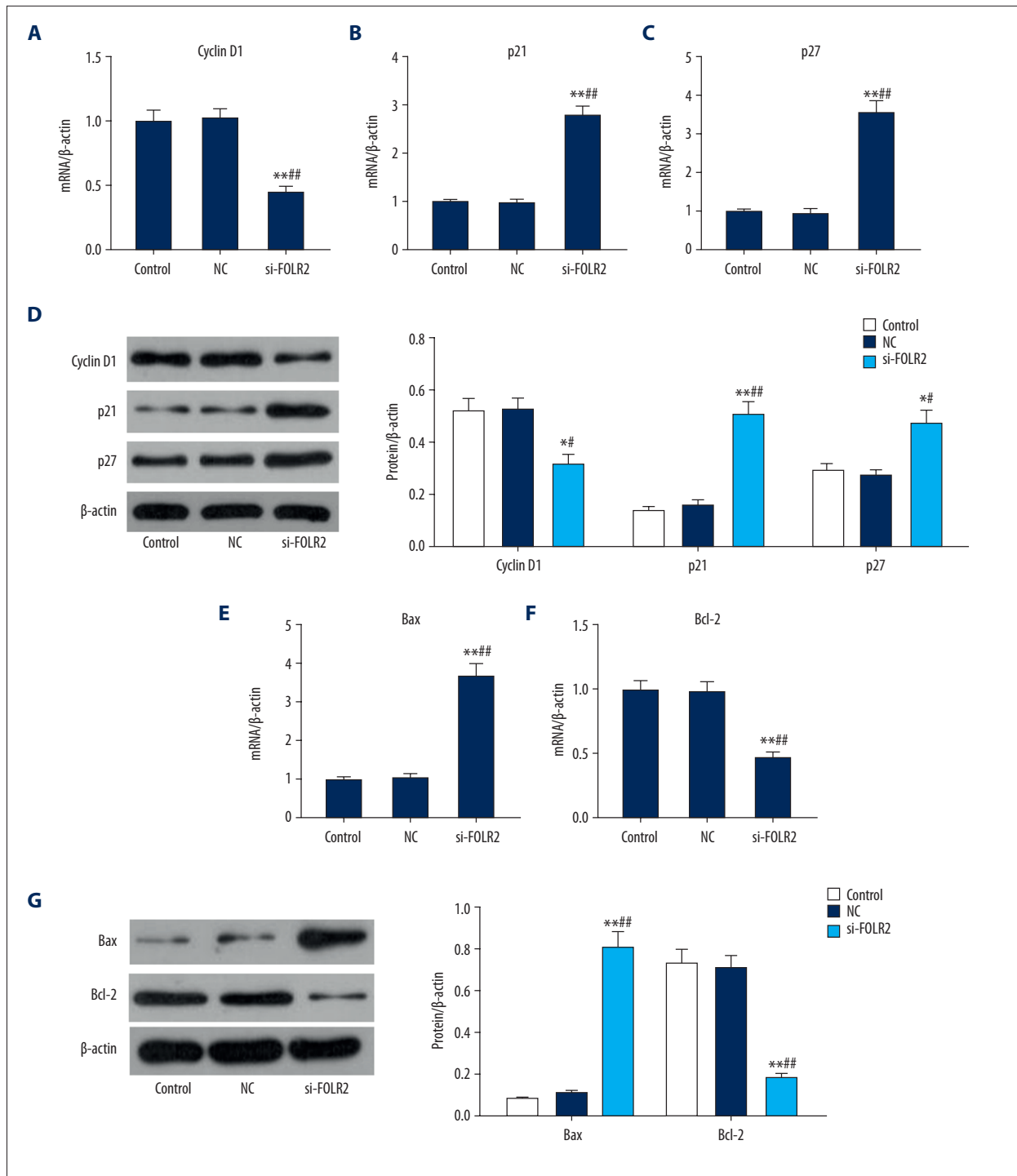


Figure 3. Silencing of expression of the *FOLR2* gene with small interfering RNA (siRNA) affected the expression of cell cycle-associated genes and apoptosis-related genes in NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the expression of mRNA for cyclin D1 (**A**), p21 (**B**), p27 (**C**), in NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells. (**D**) Western blot was used to detect the protein levels of cyclin D1, p21, and p27 in NCI-H1650 human NSCLC cells. Using qRT-PCR, mRNA expression of Bax (**E**) and Bcl-2 (**F**) were assessed in NCI-H1650 human NSCLC cells. (**G**) Western blot detected the protein levels of Bax and Bcl-2 in NCI-H1650 human NSCLC cells. β-actin was used as an internal control. Data are expressed as the mean ± SD from three independent experiments. (* Compared with control, # compared with NC, */# P<0.05; **/### P<0.01).

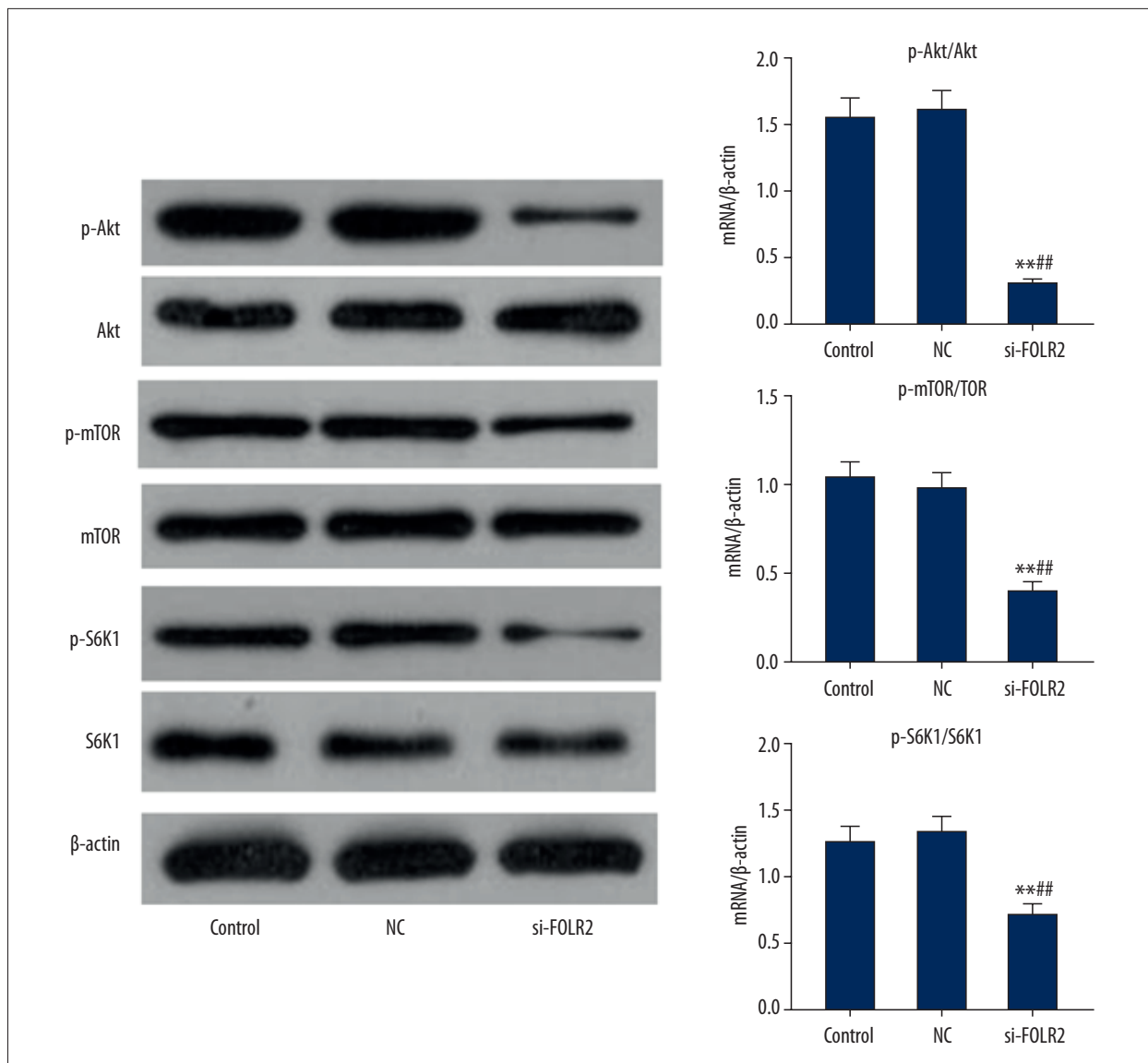


Figure 4. Protein expression levels of the AKT/mTOR/S6K1 signaling pathway in NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells. The protein expression levels of AKT, p-AKT, mTOR, p-mTOR, S6K1, p-S6K1 were detected by Western blot analysis in NCI-H1650 human non-small cell lung carcinoma (NSCLC) cells (p, phosphorylation). β -actin was used as an internal control. Data are expressed as the mean \pm SD from three independent experiments. (* Compared with control, # compared with NC, **/## P<0.05; **/### P<0.01).

Silencing of *FOLR2* expression (*siFOLR2*) arrested the cell cycle of the human NSCLC cell line, NCI-H1650

To investigate the effect on proliferation of *siFOLR2* transfected NCI-H1650 cells, the cell cycle was studied using flow cytometry. As shown in Figure 2E, the percentage of cells in the *siFOLR2* group showed a significantly increased G1 phase compared with the control or NC group (P<0.05) (Figure 2E). Both the S phase and G2 phase were significantly reduced in *siFOLR2* NCI-H1650 cells compared with the control cells (S phase, P<0.05) (G2 phase, P<0.01) (Figure 2E).

Silencing of *FOLR2* expression (*siFOLR2*) affected the expression of cell cycle-associated genes in the human NSCLC cell line, NCI-H1650

To further study how *siFOLR2* transfection in NCI-H1650 cells could affect the cell cycle, the expression of cell cycle-associated genes were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Cyclin D1 mRNA expression was significantly reduced in *siFOLR2* NCI-H1650 cells compared with the control or NC cells (P<0.01) (Figure 3A). Expression of the mRNA of the cell cycle suppressors, p21

and p27, were significantly increased in si*FOLR2* NCI-H1650 cells ($P < 0.01$) (Figure 3B, 3C). These results were partly consistent with the findings for the protein levels in si*FOLR2* NCI-H1650 cells compared with the control or NC cells, cyclin D1 ($P < 0.05$); p21 ($P < 0.01$); p27 ($P < 0.01$) (Figure 3D).

Silencing of *FOLR2* expression (si*FOLR2*) affected the expression of apoptosis-associated genes in the human NSCLC cell line, NCI-H1650

The expression of apoptosis-related genes BAX and Bcl-2 genes were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. The result showed that both mRNA and protein level for BAX were significantly increased in si*FOLR2* NCI-H1650 cells compared with the control or NC cells ($P < 0.01$) (Figure 3E, 3G). Compared with the control or NC cells, the si*FOLR2* group showed significantly reduced levels of expression of Bcl-2 mRNA and protein ($P < 0.01$) (Figure 3F, 3G).

Silencing of *FOLR2* expression (si*FOLR2*) inhibited the AKT/mTOR/S6K1 signaling pathway in the human NSCLC cell line, NCI-H1650

To investigate the molecular mechanism for the effect of si*FOLR2* on cell proliferation, the cell cycle, and apoptosis in the human NSCLC cell line, NCI-H1650, the protein levels of AKT, mTOR, and S6K1 signaling were detected by Western blotting. The blots showed that the protein of AKT, mTOR and S6K1 had no clear difference compared with control or NC cells, but the phosphorylation of the three proteins showed significantly decreased expression in si*FOLR2* NCI-H1650 cells ($P < 0.01$) (Figure 4). These findings indicated that silencing the expression of the *FOLR2* gene in the human NSCLC cell line NCI-H1650 could inhibit the AKT/mTOR/S6K1 signaling pathway.

Discussion

In the USA, and worldwide, lung cancer is the leading cause of cancer death, with non small-cell lung cancer (NSCLC) being the commonest histological type [1]. Studies continue to try to find more effective diagnostic and treatment methods for human NSCLC. The folate receptor gene *FOLR2*, a member of folate receptor family, has distinct difference with *FOLR1* which is expressed in normal tissues and several types of malignancy [27–30].

The findings of the present study demonstrated *FOLR2* expression on some human NSCLC cell lines including A549, NCI-H1299, NCI-H1650, and NCI-H460 and compared the findings in a normal human bronchial epithelial (HBE) cell line. The result showed high expression of *FOLR2* in NSCLC cell

lines compared with normal HBEs. This finding differs from previous studies that have shown that *FOLR2* and FR- β were preferentially expressed in tumor-associated macrophages (TAMS) rather than cancer cells [31,32]. However, *FOLR2* expression has been previously reported in tumor cells of myelogenous leukemia [15].

In this study, *FOLR2* gene silencing (si*FOLR2*) was performed for NCI-H1650 cells, which resulted in reduced cell proliferation and cell viability, increased cell apoptosis and inhibited the phosphorylation of AKT, mTOR, and S6K1. *FOLR2* gene silencing (si*FOLR2*) in NCI-H1650 cells also affected the cell cycle, with a significantly increased G1 phase and significantly reduced S phase and G2 phase when compared with control cells. Cell cycle-associated genes cyclin D1, p21 and p27, apoptosis-associated genes, BAX and Bcl-2 were measured. Cyclin D1 is a cell-cycle regulator that plays an important role in promoting cell cycle G1/S transformation and is also associated with tumorigenesis [33]. Also, p21 and p27 are two cyclin-dependent kinase (CDK) suppressors responsible for the regulation of the cell cycle [34]. Additionally, p21 is a known cell cycle inhibitor, which binds to cyclin-CDK complexes and proliferating cell nuclear antigen (PCNA), which induces cell cycle arrest at the G1 phase [35,36].

The findings from the present study showed that si*FOLR2* could significantly down-regulated cyclin D1 expression, with upregulation of p21 and p27 both at the mRNA and protein levels. This finding was supported by the findings of the cell cycle assay detected by flow cytometry, which showed cell cycle arrest in the G1 phase. The pro-apoptosis gene BAX showed high expression, and the apoptosis suppressor Bcl-2 showed low expression, which was consistent with the findings of the apoptosis rate in si*FOLR2* NCI-H1650 cells. These findings support that silencing of *FOLR2* expression could inhibit cell proliferation, arrest the cell cycle, and promote cell apoptosis in the human NCI-H1650 NSCLC cell line.

The AKT/mTOR/S6K1 signaling pathway has previously been reported to be a molecular mechanism involved in several human tumors including prostate cancer and breast cancer [37–39]. Phosphorylated AKT can directly activate mTOR, and also can activate mTOR through direct phosphorylation of mTOR Ser²⁴⁴⁸ sites [40,41]. Activation of AKT/mTOR signaling is associated with occurrence and development of cancer cells [42]. The results of the present study showed that si*FOLR2* significantly down-regulated the expression of p-AKT and p-mTOR in NCI-H1650 cells. S6K1 is one of the important substrates of mTOR, existing downstream of AKT/mTOR and acting as an effector of tumor cells proliferation [43,44]. This study showed that silencing the expression of *FOLR2* (si*FOLR2*) also reduced the protein level of S6K1 in NCI-H1650 cells. Therefore, it is possible to interpret from the findings of this study that si*FOLR2*

could inhibit AKT phosphorylation, which prevented mTOR activation and inhibiting S6K1 phosphorylation, which suppressed cells proliferation and induced apoptosis in the human NSCLC cell line, NCI-H1650, *in vitro*.

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Conclusions

Silencing of the *FOLR2* gene could arrest the G1 phase of the cell cycle and induce the cell apoptosis through inhibition of the AKT/mTOR/S6K1 signaling pathway in the human NSCLC cell line, NCI-H1650, *in vitro*.

Conflict of interest

None.

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