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Original article

UNC5A, an epigenetically silenced gene, functions as a tumor suppressor in non-small cell lung cancer

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ABSTRACT

UNC5A has been reported to be related with human cancers. However, the function and mechanism in non-small cell lung carcinoma (NSCLC) remains unknown. We analyzed two NSCLC cell lines (A549 and H157), one normal human bronchial epithelial cell line (BEAS-2B) and the tissues of NSCLC. We used quantitative real-time PCR (qRT-PCR), western blot and immunohistochemical (IHC) staining to examine the expression of UNC5A. Methylation status of the UNC5A promoter was analyzed using methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP). We used western blot to analyzed protein levels of PI3K/Akt pathway. We found that the mRNA expression of UNCA5 was significantly downregulated in NSCLC cells and tissues. The promoter of UNC5A was hypermethylated in NSCLC cells compared to normal control cells. The expression of UNC5A could be reversed by demethylation agent in NSCLC cells. The expression of UNC5A was decreased in NSCLC samples and significantly associated with the advanced types of NSCLC. Functionally, knockdown of UNC5A promoted cell proliferation, migration, invasion and induced apoptosis in NSCLC, overexpression of UNC5A yielded the opposite result. Moreover, we found that UNC5A negatively regulated PI3K/Akt signaling pathway in NSCLC. UNC5A is a novel epigenetically silenced gene in NSCLC and consequent under-expression of UNC5A may contribute to NSCLC tumorigenesis through regulating PI3K/Akt pathway.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer with high incidence and mortality in the world (Rulli et al., 2015). The major subtypes of NSCLC including lung adenocarcinoma (LAC) and squamous cell carcinoma (LSCC), accounts for approximately 40% and 30% of total NSCLC cases, respectively (Lemjabbar-Alaoui et al., 2015). Despite advances in treatment during the past years, the 5-year overall survival rate remains no more than 15% (Siegel et al., 2012). The molecular mechanism underlying oncogenesis and carcinogenesis of NSCLC is complex and multifaceted. Therefore, verifying of effective and novel therapeutic targets in NSCLC and exploring of the molecular biology are beneficial for the diagnosis and treatment of this disease.

The pathogenesis of NSCLC is a multi-step process, involving a variety of complicated factors, including genetic and epigenetic alterations (Schiffmann et al., 2016; Ansari and Shackelford, 2016; Pikor et al., 2013). Epigenetic gene silencing of tumor suppresser genes through promoter CpG island hypermethylation is a common character in human malignancy, including NSCLC (Jiang et al., 2014; Chen et al., 2018). For example, the low expression of METH-2 was observed in NSCLC tissues and associated with the promoter hypermethylation status (Dunn et al., 2004). The CpG island of ZAR1 promoter is hypermethylated in primary NSCLC tissues and cells, and overexpression of ZAR1 inhibited colony formation and cell cycle progression (Richter et al., 2017). The promoter region of HOXA11 was highly methylated in NSCLC cells, and HOXA11 hypermethylation was associated with Ki-67 proliferation index and pT stage of tumors (Hwang et al., 2013). Moreover, the methylated genes may be used as diagnostic and prognostic biomarkers for NSCLC (Hsu et al., 2005; Liu et al., 2017). Identification of novel targets silenced by promoter hypermethylation may provide novel insights into the mechanisms of NSCLC development.

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UNC5A is located at chromosomes 5q35 region, and belongs to a family of netrin-1 receptors. Several studies have been reported that UNC5A was involved in many types of cancer. In brain tumors, p53 could activate the expression of UNC5A by binding to the promoter region of UNC5A, and overexpression of UNC5A suppressed colony formation of glioblastoma cells (Miyamoto et al., 2010). UNC5A might act as tumor suppressor through inhibition of cell growth and regulation of cisplatin resistance in bladder cancer (Zhu et al., 2014). Activation of NF-κB in glioma by netrin-1 was dependent on UNC5A receptor and UNC5A silencing suppressed NF-κB p65 phosphorylation, c-Myc up-regulation and decreased cell proliferation (Chen et al., 2017). The expression of UNC5A was negatively correlated with EGFR expression and lower expression of UNC5A was associated with poor clinical outcome of breast cancer patients (Padua et al., 2018). However, the exact function and mechanism of UNC5A in NSCLC has not been reported yet.

In this study, we analyzed the epigenetic regulation of UNC5A in NSCLC cell lines and tissues, and the effect of overexpression or knockdown of UNC5A on cell viability and motility.

2. Materials and methods

2.1. Cell lines

Human lung cancer cell lines (SK-MES1, H358, H460, H157, H1650, A549), a human bronchial epithelial cell line (BEAS-2B) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 media containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), and 1% penicillin–streptomycin (Thermo), and were maintained at 37 °C in an atmosphere of 5% CO_2.

2.2. Patients and tissue samples

Tumor tissues and their paired adjacent non-tumor tissues (dissected at > 0.5 cm from the margin of the neoplastic lesion) were obtained from 121 NSCLC patients who had undergone surgery at The First Hospital of China Medical University. No chemo- or radio-therapy had been administered to these patients before surgical resection, and none of the patients had cancer history. The pathological stage of each samples, as well as non-tumor tissues was determined by two pathologists according to the guidelines of the AJCC TNM (Eighth) staging system. All samples were frozen immediately in liquid nitrogen and stored at −80 °C until use. Written informed consent was collected from participants before surgery, and this study was approved by the Ethical Committee of The First Hospital of China Medical University. Among these 121 cases, 41 of these cases were used in methylation and mRNA expression assay, and 80 of them were used to detect the protein expression of UNC5A.The clinicopathological features of patients were summarized in Table 1 and Table 2, respectively.

2.3. Bisulfite convertion, methylation specific PCR (MSP) and bisulfite sequencing PCR (BSP)

Genomic DNA was extracted from NSCLC cells and tissues using Qiagen DNeasy Tissue Kit (Qiagen, Düsseldorf, Germany) according to manufactures' protocol. After DNA isolation, NanoDrop 2000 (Thermo, Waltham, MA, USA) was used to measure the quality and quantity of DNA samples. Then, a total of 2 μg genomic DNA was subjected to bisulfite modification by using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions.

Both MSP and BSP primers were designed by using Methyl Primer Express v1.0 software (ABI, UK). Two primer pairs were used to detect the methylated (M) and unmethylated (UM) alleles, respectively. Each reaction contained a 100 ng of bisulfite-converted DNA, 0.2 μM of each primer, 1 μM dNTPs, 2 μl 10 × PCR buffer, and 1 unit of Taq Polymerase (Invitrogen, Carlsbad, CA, USA) in a final reaction volume of 20 μl. The primers were as follows: for methylated products: 5′-GATTGTAGCG GAATTTGTTC-3′ (forward) and 5′- ACTCCGAATCCGAACTCT-3′ (reverse); for unmethylated products: 5′- GAGGATTGTAGTG GAATTT GTTT-3′ (forward) and 5′-AATACTCCAAATCCAAACTCT-3′ (reverse). Each PCR product was stained with ethidium bromide and visualized under UV light.

Genomic DNA from A549, H157, and BEAS-2B cells were used in BSP analysis. The primers used for BSP were designed to amplify DNA spanning −620 to −275 bp upstream of the UNC5A transcription start site (TSS). The PCR products were cloned into pCR2.1 vectors according to the manufacturer's protocol (Invitrogen) and finally 5 clones for each sample were subjected to sequencing.

2.4. 5-aza-2′-deoxycytidine (5-Aza) treatments

NSCLC cells were treated with 5-Aza (Sigma, St Louis, MO, USA) at a concentration of 5 μM in the medium, which was refreshed every 24 h for three days. Cells were collected at 0, 24, 48, and 72 h and total RNA was isolated for PCR analysis.

2.5. Quantitative real-time PCR (qRT-PCR)

RNA from tissue samples and cell lines was isolated using TRIzol Reagent (Sigma), and a total of 2 mg total RNA was used to synthesize first strand cDNA using the Superscript III-reverse transcriptase kit (Invitrogen). PCR reaction was carried out using SYBR Green PCR Master Mix (ABI, Foster City, CA, USA) based on the manufacturer's protocol. ABI 7900 sequence system was used to amplification and the cycling condition is 95 °C 5 min, 40 cycles (95 °C for 30 sec, 52 °C for 20 sec and 72 °C for 20 sec), 72 °C for 10 min. β-Actin levels were used for normalization. The following primers were used for detection UNC5A: 5′- GGACACCCG CAACTGTACC-3′ (forward) and 5′-AATGGACGAGTCAGCCACATC-3′ (reverse).

2.6. SiRNA and cDNA transfection

The siRNA-1 sequence (sense) 5′-GCACCAGCAACATGACCTA-3′ and si-RNA2 sequence 5′-GCTGATGATCCCTAATACA-3′ were designed to knockdown the expression of the human UNC5A gene, and a none-specific siRNA (siRNA-NC) was used as negative control. The complete coding region of UNC5A cDNA was obtained from GeneBank and then subcloned into the lentiviral vector. Recombinant lentiviruses were produced by co-transfection 293 T cells with the packaging plasmids using Lipofectamine 2000 (Invitrogen). The cells stably expressing UNC5A vectors were named as Lv-UNC5A and the empty vectors were used as negative control (Lv-NC).

2.7. Cell proliferation assay

After siRNA or lentivirus infection, NSCLC cells were plated in 96-well plate at a density of 2×10^3 cells per well, and cell viability was measured at 24, 48, and 72 h using the methylthiazoletetrazolium (MTT) assay kit (Promega, Madison, Wisconsin, USA). Absorbance was measured on a microplate reader (Thermo) at a wavelength of 490 nm.

2.8. Flow cytometry for cell cycle and apoptosis analysis

Flow cytometry was utilized to detect the cell cycle and apoptosis. Cells were seeded in 6-well plates and cultured for 48 h and

Table 1
The correlation between UNC5A methylation or expression and clinicopathological features.

Characteristic	No. case	UNC5A methylation		p value	UNC5A mRNA		p value
	n = 41	Methylated	Unmethylated		High	Low	
Age (years)				0.547			0.423
≥59	25	18	7		12	13	
<59	16	11	5		9	7	
Gender				0.328			0.471
Male	34	25	9		18	16	
Female	7	4	3		3	4	
Smoking History				0.380			0.191
Yes	27	20	7		12	15	
No	14	9	5		9	5	
Histology				0.495			0.595
Adenocarcinoma	12	8	4		6	6	
Squamous cell carcinoma	29	21	8		15	14	
Tumor size				0.152			0.020
<5 cm	24	15	9		16	8	
≥5 cm	17	14	3		5	12	
TNM stage				0.021			0.009
I,II	19	10	9		14	5	
III,IV	22	19	3		7	15	
Lymph node metastasis				0.160			0.093
No	11	6	5		8	3	
Yes	30	23	7		13	17	

Table 2
The correlation between UNC5A protein levels and clinicopathological features.

Features	No. Case n = 80	Negative n = 53	Positive n = 27	p
Gender				
Male	66	44	22	0.865
Female	14	9	5	
Age (years)				
<62	35	26	9	0.183
≥62	45	27	8	
Histology				
SCC	58	34	24	0.020
AC	22	19	3	
TNM stages				
IIA	5	0	5	0.000
IIB	16	5	11	
IIIA	28	22	6	
IIIB	31	26	5	
Smoke				
Yes	67	44	23	1.000
No	13	9	4	
KPS grade				
≥90	65	39	26	0.015
<90	15	14	1	

SCC, squamous cell carcinoma; AC, adenocarcinoma.

then were collected with trypsin digestion solution, washed twice PBS. For cell cycle analysis, cells were resuspended in 200 μl PBS with 10 μl propidium iodide (PI) and incubated at room temperature in the dark for 15 min. For cell apoptosis analysis, cells (1×10^5 cells/well) were seeded in 6 well-plate, then washed with cold PBS and centrifuged at 2000 rpn for 10 min to resuspend in binding buffer. Then 5 μl Annexin V-FITC was added and incubated for 15 min in the dark. 5 μl propidium iodide (PI) was added to the cells before analyzed. Flow cytometry analysis was carried out using a FACS can (Beckman Coulter, Fullerton, CA, USA).

2.9. Wound-healing and transwell invasion assay

A scratch wound-healing assay was performed to assess cell mobility. Cells were seeded in 6-well plates at a density of 1×10^6 cells/well in RPMI-1640 medium. The cell monolayer was wounded by scratching a line with sterile 200 μl pipette tip. The detached cells were removed by washing the cell monolayer with PBS for 10 sec using a sterile rubber dropper. The images were captured at 0 and 36 h using a digital camera.

The invasive ability of cells were measured by transwell assay. The transfected cells (2×10^5) cells were seeded in the upper chambers of Matrigel-precoated Transwell plates (Corning, Lowell, CA, USA). Lower chambers were filled with medium containing 10% FBS. After 48 h incubation, the cells in the lower surface of the filter were fixed, stained by 2% crystal violet, and counted using a light microscope in 5 randomly selected fields.

2.10. Immunohistochemistry (IHC) assay

Immunohisto-chemical staining was performed to evaluate the protein levels of UNC5A in clinical samples. The staining intensity scores were divided into four groups: no staining marked 0; weak marked 1; moderate marked 2, and strong marked 3. For the percentage of positively stained cells, we divided them into groups labeled from 0 to 4 (<5% labeled 0; 5%-25% labeled 1; 25%-50%

labeled 2; 51%-75% labeled 3; 75% labeled 4). The expression of UNC5A was obtained by multiplying the intensity fraction and the percentage area. After making the calculations, a cut-off value of 5 was selected since the median score of UNC5A expression in NSCLC was 5 in the present study. we separated the specimens according to expression into two groups (negative expression and positive expression), 0 and 5 were evaluated as negative expression, 6–12 were higher expression.

2.11. Western blot analysis

Cells were harvested and lysed using RIPA buffer and protein concentrations were determined using the BCA protein assay kit (Thermo). Then equal amounts of proteins were separated on 10% SDS-PAGE and then were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin in TBST and then incubated in primary antibodies overnight at 4 °C followed by secondary antibodies for 1 h at 37 °C. Primary antibodies AKT, p-AKT, PI3K, p-PI3K, UNC5A, p21, and Cyclin D1 were purchased from Abcam (Cambridge, UK). The signal was visualized through a chemiluminescent detection system (Thermo).

2.12. Statistical analysis

The data were presented as the mean ± standard error of mean (SEM) and Prism 5 (GraphPad Software, San Diego, CA, USA) was used to analysis experiment data and generate graphs. Chi-squared test or Fisher's exact test were used to analyze correlations between UNC5A methylation or expression and clinicopathological features of patients. UNC5A methylation or expression levels between tumors and non-tumor tissues were compared using the paired-samples *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. UNC5A promoter CpG island was methylated in NSCLC cells

In order to know the expression of UNC5A in NSCLC, we firstly evaluated the mRNA levels of UNC5A in NSCLC cells. As shown in Fig. 1A, The expression of UNC5A was downregulated in NSCLC cells compared to adjacent non-tumor tissues. Moreover, the mRNA levels of UNC5A is significantly low in NSCLC tissues (0.034 ± 0.023) than that in non-tumor tissues (0.094 ± 0.063, $p < 0.001$, Fig. 1B).

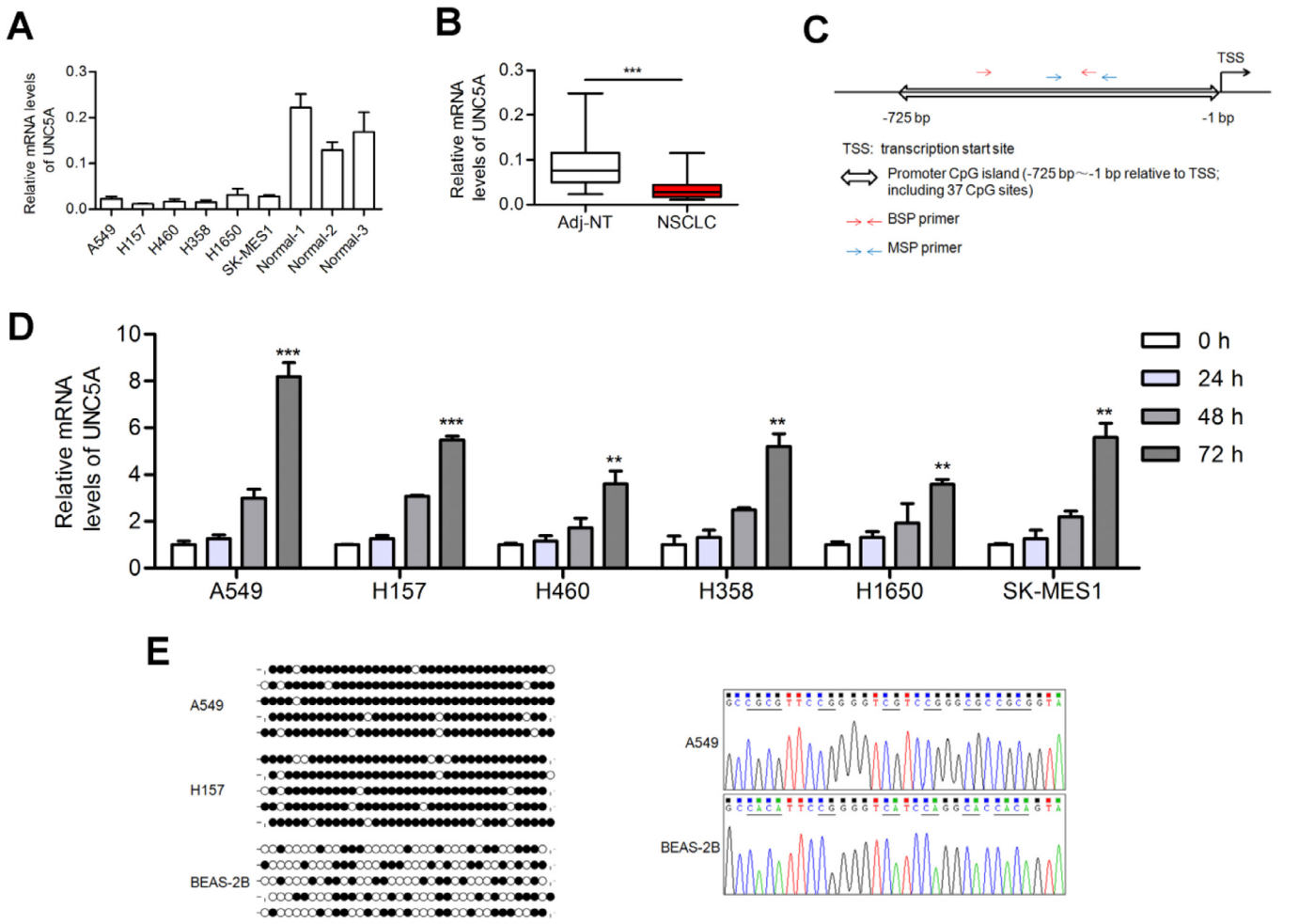

Fig. 1. The CpG island of UNC5A promoter was hypermethylated in NSCLC cells. (A) The expression of UNC5A was downregulated in NSCLC cells. (B) The mRNA levels of UNC5A was higher in non-tumor tissues than that in tumors. (C) A CpG island was located in UNC5A promoter region. (D) The mRNA levels of UNC5A was restored by 5-Aza treatment for 72 h in NSCLC cells. (E) The methylation status of 37 CpG sites in the promoter region of UNC5A was determined by BSP assay (left). Black dot, methylated CpG sites; white dot, unmethylated CpG sites; stub, not available; Representative sequences of BSP analysis in A549 cells and BEAS-2B cells (right). Adj-NT, adjacent non-tumor tissues; **, $P < 0.01$; ***, $P < 0.001$.

By using database UCSC, we found a CpG island in promoter region of UNC5A (Fig. 1C), suggesting that promoter hypermethylation may be an explanation for UNC5A downregulation. Consideration that gene expression could be reversed by demethylation agent, the mRNA expression of UNC5A in NSCLC was evaluated by qRT-PCR after 5-Aza treatment. The mRNA expression of UNC5A was increased several fold in the 5-Aza treated cells at 72 h compared with that in the control cells at 0 h (Fig. 1D). These results suggested that hypermethylation may be responsible for down-expression of UNC5A in NSCLC cells. The BSP assay was used to validate the methylation status of UNC5A promoter CpG island. The CpG island (−620 to −275) including 37 CpG sites was amplified by PCR and sequenced. Representative results are shown in Fig. 1E. The 37 CpG sites in this region were identified to be hypermethylated in NSCLC cell lines, while only partially methylated in BEAS-2B cells. These results confirmed the UNC5A promoter hypermethylation status in NSCLC cells.

3.2. The methylation and expression of UNC5A in clinical samples

In order to check the methylation status of UNC5A in clinical samples, MSP assay was conducted in 41 NSCLC tissues and the paired noncancerous tissues. As shown in Fig. 2A, UNC5A promoter region was frequently methylated in tumor tissues than that in non-tumor tissues. The immunochemical staining of UNC5A protein in primary NSCLC tissues were shown in Fig. 2B. 53 (66.3%) of the 80 primary NSCLC tissue samples were negative for UNC5A expression, while the other 27 (33.7%) were positive expression of UNC5A.

The correlations between methylation and expression of UNC5A and clinocopathological parameters were evaluated and the results were shown in Table 1. The methylation UNC5A promoter was positively correlated with tumor stage ($p = 0.021$). The median level of UNC5A mRNA levels in tumors was used as cut-off value to devided cases into two groups (high or low group), the low expression UNC5A was significantly associated with the larger tumor size ($p = 0.02$) and advamced TNM stage ($p = 0.009$, Table 1). Besides, the low protein expression of UNC5A indicates the aggressive phenotypes of NSCLC ($p < 0.001$, Table 2).

3.3. UNC5A inhibits cell growth and induces apoptosis

To determine the function of UNC5A in NSCLC development, we firstly knockdown of UNC5A in NSCLC cells by siRNA transfection method. The qRT-PCR was conducted and the data confirmed that the expression of UNC5A was noticeably reduced in si-RNA1 transfected cells (Fig. 3A) compared to si-NC transfected cells. For cell proliferation assay, we found that the proliferation rates were significantly increased in si-RNA1 treated NSCLC cells (Fig. 3B). Then, we found that cells at S phase was significantly increased in UNC5A silencing cells (Fig. 3C). Apoptosis analysis demonstrated that knockdown of UNC5A reduced the number of apoptotic cells (Fig. 3D).

To determine if increased UNC5A expression changes cellular processes, we generated stable NSCLC cells that expressed either UNC5A vector (Lv-UNC5A) or negative control vector (Lv-NC). As shown in Fig. 4A, the transfection efficiency was determined by qRT-PCR assay and the results showed that transfection with

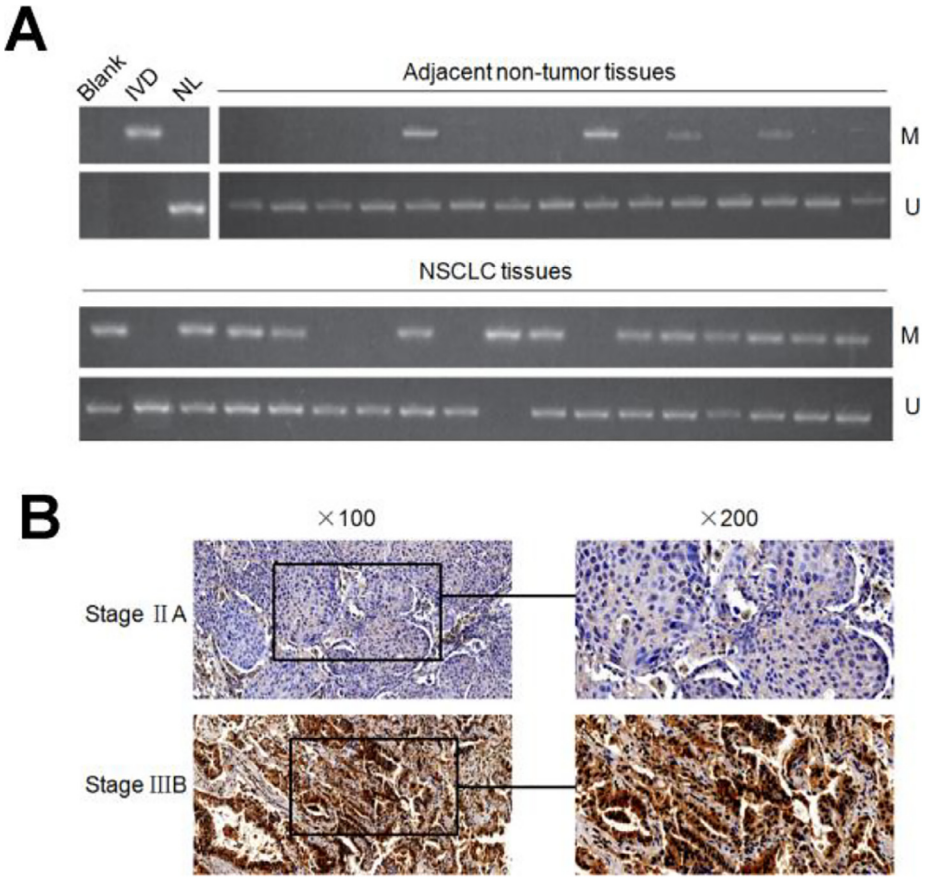

Fig. 2. The methylation and protein expression of UNC5A in clinical samples. (A) The methylation status of UNC5A promoter CpG island in tissue samples (n = 41) was tested by MSP methyod; M, methylated products; U, unmethylated products. (B) Immunohistochemistry analysis of UNC5A protein expression in NSCLC tissues (n = 80).

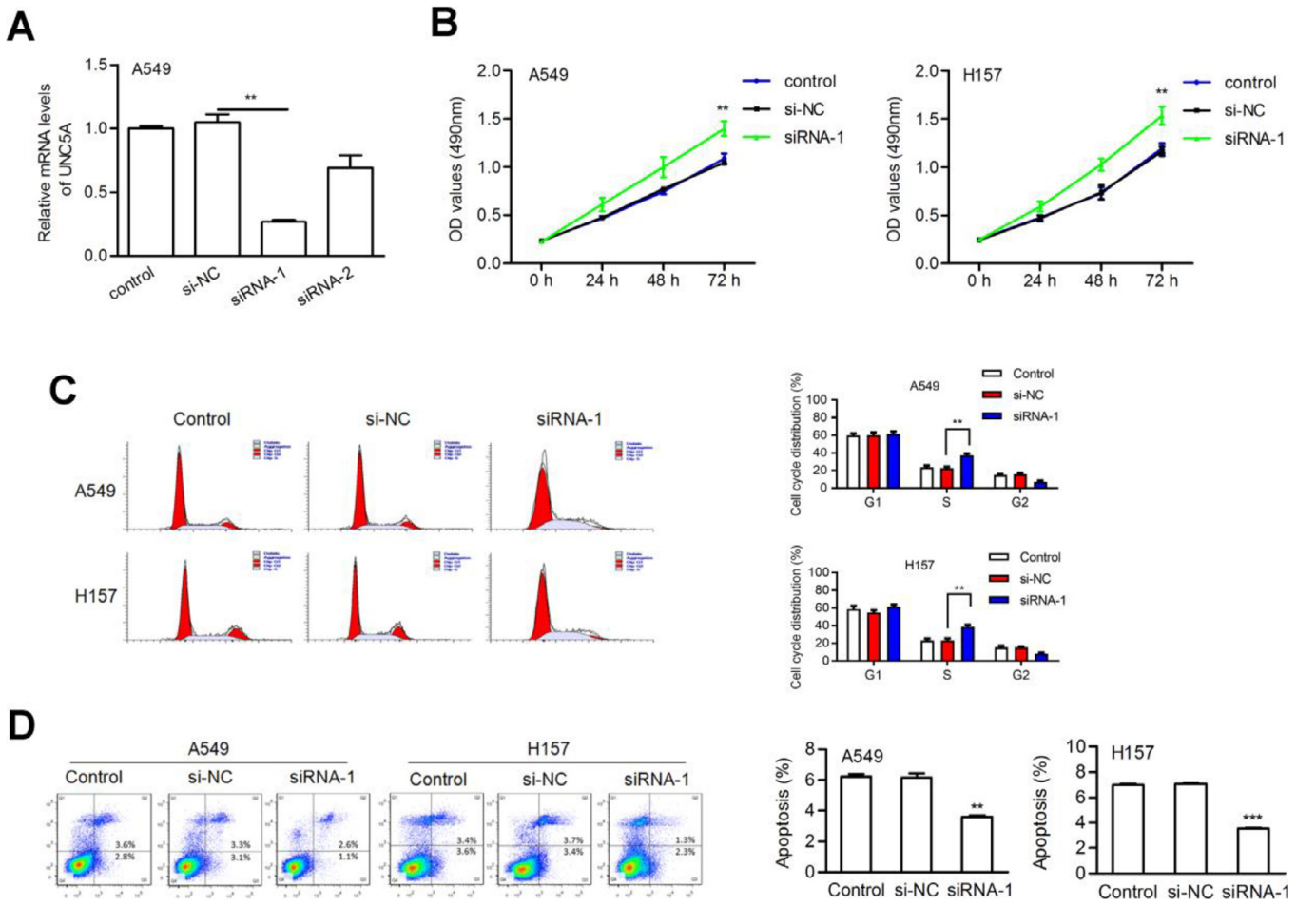

Fig. 3. The Effect of UNC5A on cell proliferation, cell cycle distribution, and apoptosis. (A) Transfection of si-RNA1 decreased the expression of UNC5A in NSCLC cells compared to negative control group. (B) Knockdown of UNC5A promoted cell proliferation. (C) UNC5A silencing cotributed to cell cycle progression. (D) The number of apoptotic cells was decreased in A549 and H157 cells after transfection with si-RNA1. **, $P < 0.01$; ***, $P < 0.001$; control, blank control group; si-NC, siRNA negative control; si-RNA1, siRNA for knocking down UNC5A group.

UNC5A vectors led to an significant upregulation of UNC5A mRNA expression. Cell proliferation was attenuated in A549 and H157 cells stably expressing UNC5A compared to cells transfected with empty vector (Fig. 4B). Ectopic expression of UNC5A decreased the cells at S phase compared to control cells (Fig. 4C). UNC5A overexpression led to an increasing of apoptotic cells (Fig. 4D). All these data suggested that UNC5A inhibited cell growth through inducing cell cycle arrest and enhancing apoptosis in NSCLC.

3.4. UNC5A suppressed cell migration and invasion

The effect of UNC5A on cell motility was also evaluated. The transwell invasion assays demonstrated that the invasive of NSCLC cells were markedly enhanced after knocking down UNC5A, while decreased by UNC5A overexpression (Fig. 5A). Likewise, silencing of UNC5A promoted cell migration, while upregulation of UNC5A displayed the opposite effect (Fig. 5B). Our data indicated the inhibtory role of UNC5A on cell movement.Fig. 6.

3.5. Silencing of UNC5A activates PI3K/Akt signaling pathway

The activity of the PI3K/Akt signaling pathway was determined by western blot analysis. Firstly, the expression of UNC5A after transfection with siRNA-1 or Lv-UNC5A was validated by western blot (Fig. 5A). It was observed that following the knockdown of UNC5A, the levels of p-Akt, and p-PI3K were increased, while the total protein levels remained unchanged. Ectopic expression of UNC5A decreased the protein levels of p-Akt and p-PI3K (Fig. 5B). Moreover, the expresion of p21 was silenced by UNC5A knockdown, while induced by UNC5A expressing vectors. We also found that that the major cell cycle related protein, Cyclin D1, was upregulated following the knockdown of UNC5A, and downregulated by the overexpression of UNC5A in NSCLC cells (Fig. 5B). Furthermore, we found that PI3K inhibitor, Y294002, decreased the activity of PI3K/Akt signaling pathway induced by UNC5A knockdown (Fig. 5C). These data suggest that the PI3K/Akt signaling cascade is inactivated by UNC5A in NSCLC.

4. Discussion

Increasing studies have been reported that promoter hypermethylation of tumor suppressor gene may be used as a sensitive marker for NSCLC early diagnosis, prognosis prediction, and potential therapeutic target (Hulbert et al., 2017; Feng et al., 2016; Han et al., 2016). In our study, UNC5A is firstly demonstrated as tumor suppressor in the development of NSCLC. The expression of UNC5A was significantly down-expressed in NSCLC cells and tissues, suggesting that UNC5A may have roles in NSCLC progression. The molecular mechanism of UNC5A downregulation has attracted our attention. We found a CpG island was located in the prmoter region of UNC5A, implying the possibility of epigenetic regulation of UNC5A. The expression of epigenetically silenced gene could be

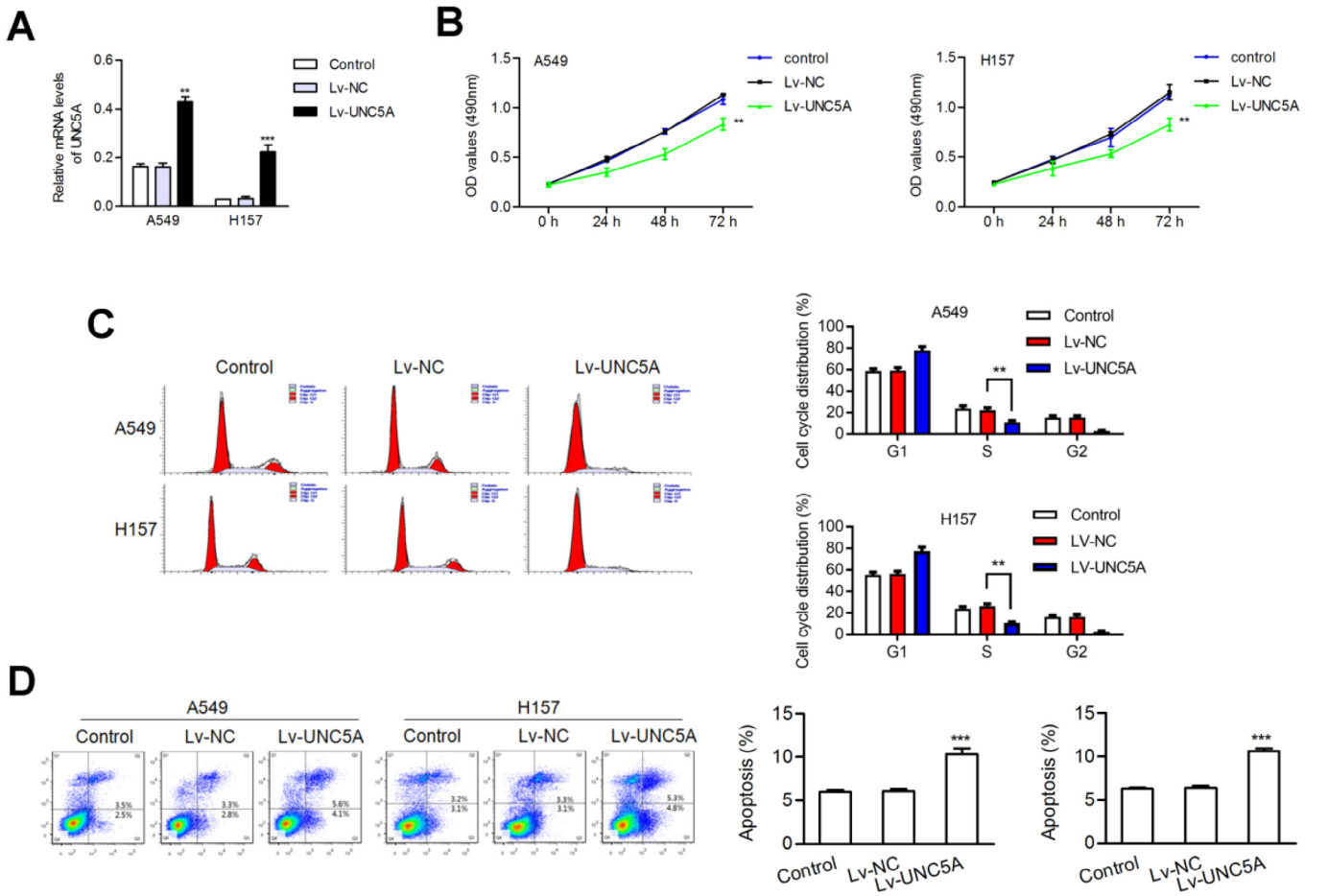

Fig. 4. Ectopic expression of UNC5A exerted tumor suppressive role. (A) The expression of UNC5A was determined by qRT-PCR assay. (B and C) Upregulation of UNC5A inhibited cell proliferation and induced cell cycle arrest. (D) Overexpression of UNC5A increased cell apoptosis. **, P < 0.01; ***, P < 0.001; Lv-NC, empty vector control; Lv-UNC5A, UNC5A expressing group.

recovered by demethylation agent 5-Aza (Deng et al., 2018; Karakoula et al., 2014; Yamamura et al., 2013). Thus, we measured UNC5A expression in NSCLC cells cultured in the presence of 5-Aza. The results showed that the mRNA levels of UNC5A in NSCLC cells were significantly restored by 5-Aza. Subsequently, BSP assay was conducted in A549, H157, and BEAS-2B cells to verify the methylation status of UNC5A promoter CpG island. We found that the CpG sites were noticeably methylated in A549 and H157 cells, while only partially methylated in BEAS-2B cells. These results confirmed that promoter hypermethylation may responsible for the downregulation of UNC5A in NSCLC cells.

The methylation status of UNC5A promoter CpG island was also detected in NSCLC tissues and non-tumor tissues. The results of MSP assay demonstrated that the methylated UNC5A was more frequently observed in NSCLC tissues compared to normal control tissues, indicating that methylation of UNC5A may be a common phenomenon in NSCLC oncogenesis. Additionally, the mRNA and protein levels of UNC5A were also measured in these clinical samples. Both the mRNA and protein expression of UNC5A were markedly decreased in tumors, suggesting that methylation of UNC5A may result in its under-expression in NSCLC cases. Moreover, methylated UNC5A indicated the advanced types of NSCLC, and UNC5A mRNA was associated with tumor size and stage, further confirmation that epigenetic regulation of UNC5A involved in lung cancer carcinogenesis. The other members of netrin-1 receptor family, such as UNC5C was also reported to be related with tumorigenesis. For example, UNC5C expression is reduced in human colorectal cancers, mainly through promoter hypermethylation (Bernet et al., 2007; Hibi et al., 2009a, 2009b; Shin et al., 2007). Loss of heterozygosity and DNA methylation led to an inactivation of UNC5C in renal cell carcinoma (Lv et al., 2011). The DNA hypermethylation of UNC5C was also observed in gastric cancer and hepatocellular carcinoma (Hibi et al., 2009a, 2009b, 2012). UNC5A was identified as a tumor suppressor that could be inactivated by DNA methylation in colorectal cancer (Okazaki et al., 2012). Thus, DNA methylation induced downregulation of netrin-1 receptor family genes might a prevalence phenomenon in various human tumors.

Functionally, the biological function of UNC5A was revealed by gain- or loss-of-function analysis. We found that silencing of UNC5A increased cell proliferation, cell cycle progression, migration, and invasion, but decreased cell apoptosis in NSCLC cells. Ectopic expression of UNC5A inhibites cell growth and motility, and enhances apoptosis. These evaidence strongly indicated the tumor suppressive role of UNC5A in NSCLC. Netrin-1-induced cell proliferation was partially attenuated by silencing of UNC5A in glioma. Silencing the endogenous UNC5A reduced cisplatin-mediated cell death and colony formation in bladder cancer. UNC5A dramatically induced apoptosis through the activation of caspase-3 in several cell lines of colon cancer and brain tumors. We speculated that UNC5A may play different roles in cancers due to the complexity of molecular mechanisms. In our study, PI3K/Akt signaling pathway was activated by UNC5A overexpression, while inactivated by UNC5A knockdown. PI3K has been shown to play a critical role in cell proliferation and anti-

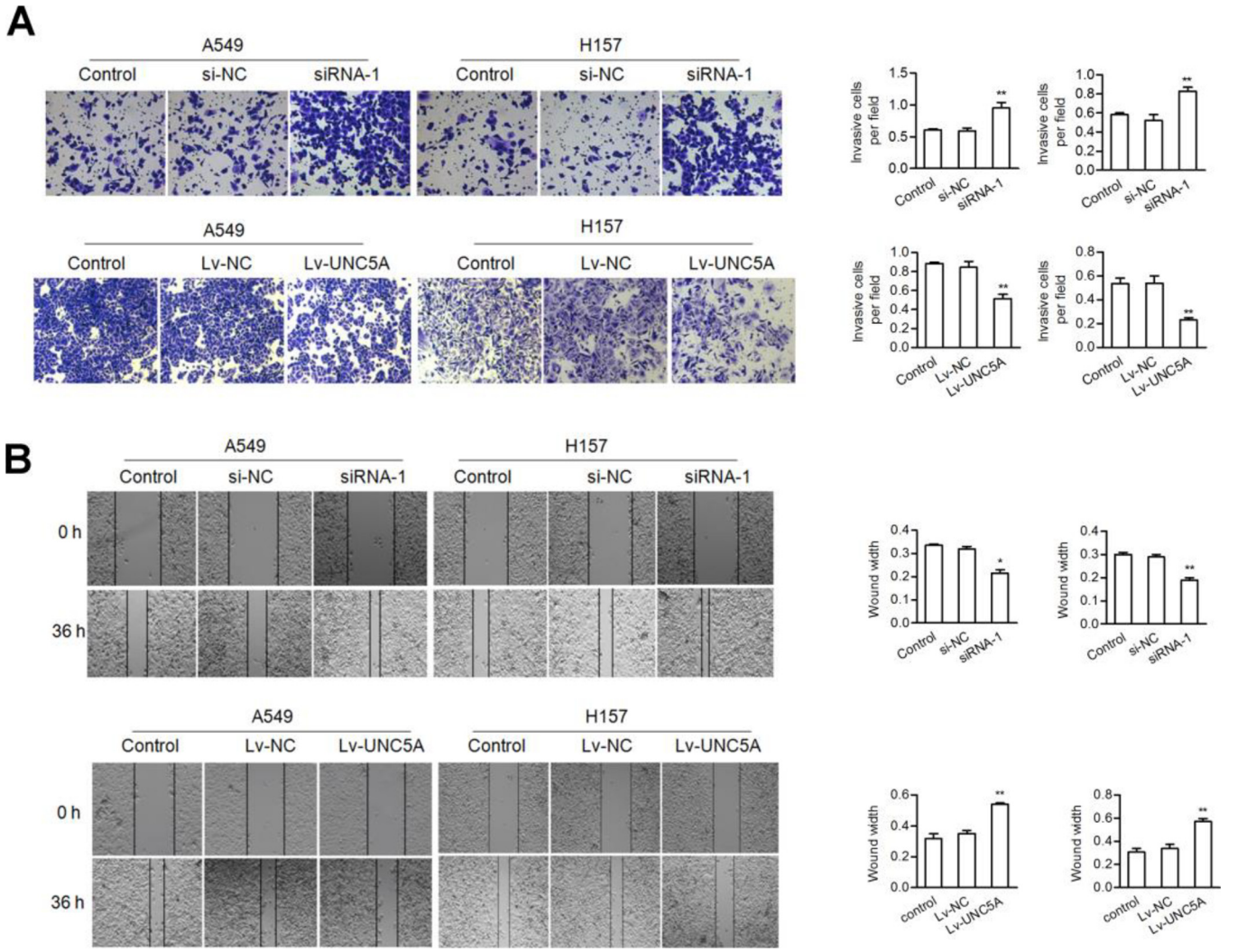

Fig. 5. UNC5A suppressed cell motility. (A) Transwell invasion assay was performed to measure cell invasive ability. (B) The migratory ability of NSCLC cells was enhanced by siRNA-1, while attenuated by Lv-UNC5A vectors transfection. *, P < 0.05; **, P < 0.01.

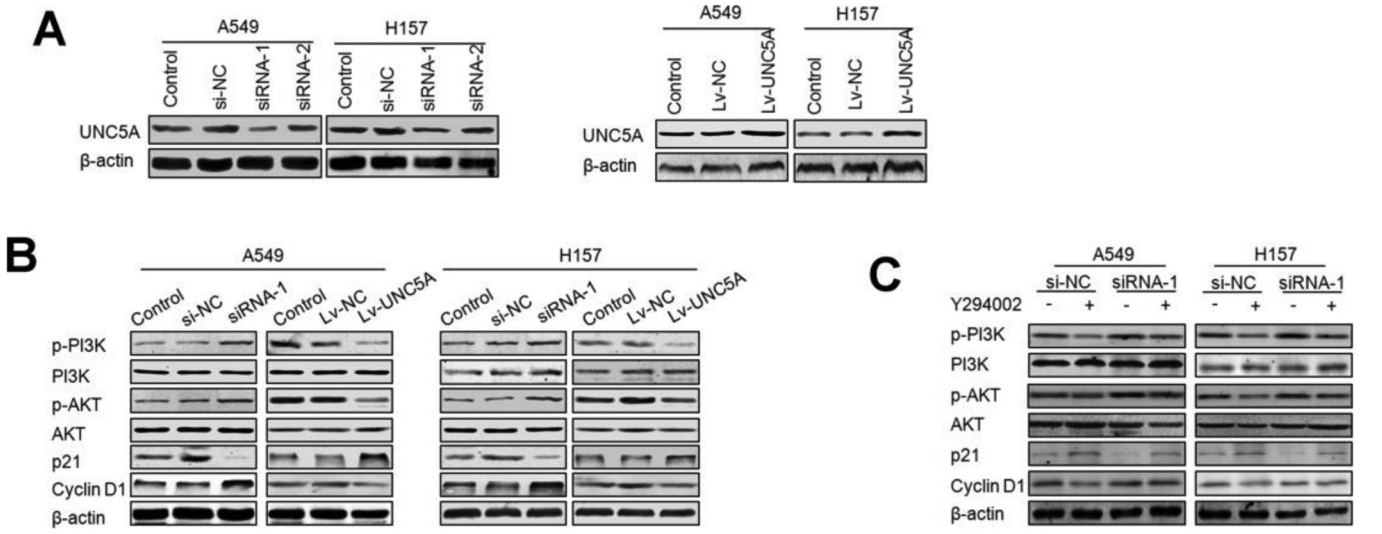

Fig. 6. UNC5A negatively regulates PI3K/Akt signaling pathway. (A) The expression of UNC5A was determined. (B) Western blot analysis of the proteins involved in the PI3K/Akt signaling cascade and downstream genes in cells. (C) PI3K inhibitor, Y294002, decreased the protein expression of p-PI3K, p-Akt, and Cyclin D1, while increased p21 expression in NSCLC cells. control, blank control; si-NC, siRNA negative control; Lv-NC, empty vector control, Lv-UNC5A, UNC5A expressing vector; siRNA-1, specific siRNA for knocking down UNC5A.

apoptosis, ultimately mediate cancer development (Andrew et al., 1999). Akt is one of the most important downstream targets of PI3K, and regulates a variety of cellular processes including cell growth, differentiation, and motility (Osaki and Oshimura, 2004; Guo et al., 2017; Liang et al., 2018; Nie et al., 2017; Wang et al., 2017). Our data suggested that UNC5A exerted tumor suppressive role in NSCLC through regulating PI3K/Akt signaling pathway.

In conclusion, this is the first study to elaborate UNC5A as a novel epigenetic silenced gene in NSCLC. Moreover, our findings highlight that the promoter hypermethylation contributes to low expression of UNC5A, thereby activating PI3K/Akt signaling pathway and promoting lung carcinogenesis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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