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microRNA-181b suppresses the metastasis of lung cancer cells by targeting sex determining region Y-related high mobility group-box 6 (Sox6)

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ABSTRACT

Background: The aim of the study was to measure the expression of microRNA (miR)-181b in patients with lung cancer, investigate its biological function and elucidate the underlying mechanisms associated with the development of lung cancer.

Methods: miR-181b expression in tissues was measured via RT-qPCR. After A549 cells were transfected with miR-181b mimic or si-Sox6, the proliferation, migration and cell cycle distribution of A549 were evaluated using cell counting kit-8 assay, transwell assay and flow cytometry. The levels of cell cycle-related proteins and Sox6 were analyzed by western blotting. Gene targets of miR-181b were predicted via bioinformatics analysis and verified using a dual-luciferase reporter gene assay.

Results: Expression of miR-181b was significantly downregulated in lung cancer tissues ($P < 0.05$), and was inversely correlated with the degree of cell differentiation and clinical stages of lung cancer (both $P < 0.05$). Additionally, the expression of miR-181b was significantly lower in adenocarcinoma compared with squamous cell carcinoma in the lungs ($P < 0.05$). Overexpression of miR-181b significantly decreased the protein level of Sox6 and significantly suppressed the cell proliferation and metastasis (both $P < 0.05$); this effect was also observed in A549 cells transfected with si-Sox6. The luciferase activity of a Sox6 3′-untranslated region-based reporter construct was significantly lower when transfected with miR-181b ($P < 0.05$), which suggests that Sox6 is a direct target of miR-181b.

Conclusion: The results of the present study suggest that miR-181b may function as a tumor inhibitor in the development of lung cancer via targeting Sox6 to decrease the proliferation and metastasis of lung cancer cells.

1. Introduction

Lung cancer is one of the most prevalent forms of cancer, the tumors of which typically include two pathological tissue types; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1,2]. SCLC accounts for ~15% of lung cancer cases, whereas NSCLC, which primarily consists of adenocarcinoma and squamous cell lung cancers, accounts for ~80% of the total number of lung cancer cases [3,4]. It has recently been reported that of malignant tumors, lung cancer has the highest mortality and, despite advances in surgery and combined therapy, the 5-year survival rate remains between 15 and 30% [5]. Tumor invasion and metastasis are largely responsible for the poor prognosis of patients with lung cancer [6,7] and are associated with various genes and multiple stages of lung cancer development; however, their underlying mechanisms remain to be elucidated. Therefore, studies on the molecular mechanisms of lung cancer invasion and metastasis may have important clinical significance.

MicroRNAs (miRNAs or miRs) are a class of post-transcriptional gene regulatory factors with lengths of 18–22 nt [8,9], which serve important roles in the development of tumor invasion and metastasis, angiogenesis and other processes [10–13]. Recent studies have indicated that the miR-181 family is also associated with tumor development [14,15]. Previous studies had revealed that miR-181b expression was altered in numerous types of cancer, and the expression pattern was cell specific. The miR-181b was upregulated expression in hepatocellular carcinoma [16]. Downregulation of miR-181b had been detected in lung cancer and prostate cancer, while upregulation of miR-181b had been demonstrated in acute myeloid leukemia and oral carcinoma [17,18]. However, the expression profile and biological function of miR-181b in the development of lung cancer are yet to be

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determined.

The aim of the present study was to measure the expression of miR-181b in patients with lung cancer, investigate its biological function and elucidate its underlying mechanisms associated with the development of lung cancer.

2. Material and methods

2.1. Lung cancer tissue collection

A total of 35 samples of human lung cancer tissues and their matched adjacent noncancerous tissues were collected between February and November 2017 at the Third Affiliated Hospital of Soochow University.., including 24 cases of adenocarcinoma and 11 cases of squamous cell carcinoma, and 18 cases of SCLC. The present study was approved by the ethics review board of the Third Affiliated Hospital of Soochow University, and written and informed consent was obtained from all patients prior to commencement.

2.2. Cell culture and transfection

A549 cells were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. HEK293 T cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated feral bovine serum and 1% penicillin-streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO2, passaged when confluence reached 90% and subsequently transfected with 25 pmol of mature miR-181b mimic (Guangzhou RiboBio Co., Ltd., Guangzhou, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. As a control group, A549 cells were transfected with a negative control mimic (NC). To silence the expression of sex determining region Y-related high mobility group-box 6 (Sox-6), cells were transfected with Sox6 small interfering (si) RNA (si-Sox6) or NC (Guangzhou RiboBio Co., Ltd.). Cells were collected 24 or 48 h post-transfection for further experimentation.

2.3. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Lung cancer tissues were ground to a fine powder in liquid nitrogen using a porcelain mortar and pestle. The total RNA was isolated using TRIzol isolation reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's instruction, and miRNA was reverse transcribed using PrimeScript RT-PCR Kit (Clontech Laboratories, Inc., Mountainview, CA, USA) following the addition of a poly (A) tail. The expression of small nuclear U6 was used as an internal control. qPCR was determined using a SYBR PrimeScript RT-PCR Kit (Takara Biotechnology Co., Ltd., Dalian, China). The primers used for miR-181b were as follows: Forward, 5′-ACATTCATTGCTGTCGGT-3′; reverse, provided by the kit. Each sample was detected in triplicate. Relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method normalized to GAPDH.

2.4. Cell counting kit-8 (CCK-8) assay

A549 cells transfected with miR-181b mimic or NC were seeded into a 96-well plate at the concentration of 1×10^3 cells in triplicate. CCK8 (Shanghai Biyuntian Bio-Technology Co., Ltd., Shanghai, China) was added to the wells at 24 h and incubated for 30 min. The absorbance value of each well was measured on a microplate reader (SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA) at 450 nm to generate proliferation curves.

2.5. Migration and invasion assay

A total of 1×10^5 A549 cells in 200 µl of DMEM were seeded in the top chamber of 8 µm-pore filter Transwell chamber inserts (Corning Incorporated, Corning, NY, USA) in 24-well plates. RPMI plus 10% fetal bovine serum (500 µl) was used as placed into the bottom chamber and incubated at 37 °C in an atmosphere containing 5% CO2. For analysis of invasive capacity, Matrigel (BD Biosciences, San Jose, CA, USA) was melted at 4 °C overnight and diluted with DMEM at 1:2. The Transwell migration chambers were coated with Matrigel on ice and stored at 37 °C for 60 min, allowing it to solidify. Cells that had not migrated or invaded and remained on the top of the membrane were removed with a cotton swab; whereas cells that did pass through and were located on the lower side of the chamber, were fixed with formaldehyde, stained with Giemsa, and were counted in five randomly selected fields using an Olympus BX51/61 light microscope (magnification, x200; Olympus Corporation, Tokyo, Japan).

2.6. Flow cytometry analysis of cell apoptosis and cycle

The flow cytometry assay was performed to measure cell apoptosis and cell cycle distribution. The apoptosis of cells were detected by Annexin V-FITC/PI apoptosis double staining kit (Becton, Dickinson and Company, NJ, USA). The cells were washed twice, and a blank group, a FITC single staining group, a PI single staining group, and a double staining treatment group (FITC and PI double staining) were separately yet. Add the dye group and incubate for 30 min at room temperature in the dark. Flow cytometry analysis was performed within 1 h, and 10^4 cells were detected in each group.

At 24 h post-transfection, $1x10^6$ cells were washed twice with cold PBS at 4°C and stained with a Cell Cycle Assay kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Cells were analyzed by flow cytometry using FACS Verse (BD Biosciences) and Modfit software (Verity Software House, Inc., Topsham, ME, USA).

2.7. Western blot analysis

Cells were washed twice with cold PBS and incubated with radioimmunoprecipitation assay buffer and protease inhibitor phenylmethane sulfonyl fluoride (Gibco; Thermo Fisher Scientific, Inc.) to extract proteins. Proteins (35 µg per lane) were subsequently denatured, separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). Membranes were subsequently blocked with skimmed milk for 1 h and incubated with the following primary antibodies: Rabbit anti-human Sox6 (1:1000; ab30455; Abcam PLC, Cambridge, England), anti-human Cyclin D1 (1:1000, 2922; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-human Cyclin E1 (1:1000, 4129; Cell Signaling Technology, Inc.). The membrane was subsequently incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies at room temperature for 1 h. The Signals detection were performed using chemiluminescence (E-001, Hanheng Bio-Technology Co., Ltd., Hong Kong, China).

2.8. Dual-luciferase reporter gene assay

According to the results of bioinformatics prediction, luciferase reporter plasmids were generated by insertion of wild type or mutant 3′UTR sequences of Sox6 into the multiple cloning site (Spe-1 and HindIII) downstream of the luciferase reporter gene in the Luciferase pMIR-REPORT miRNA Expression Reporter Vector System (Thermo Fisher Scientific, Inc.). HEK293 T cells were transfected with 0.5 µg constructed luciferase reporters and 10 ng pMIR-REPORT β-gal control plasmid as an internal control. miRNA mimics or NC RNA were also transfected. The luciferase activity was measured 24 h post-transfection using a dual-luciferase detection kit (Shanghai Biyuntian Bio-

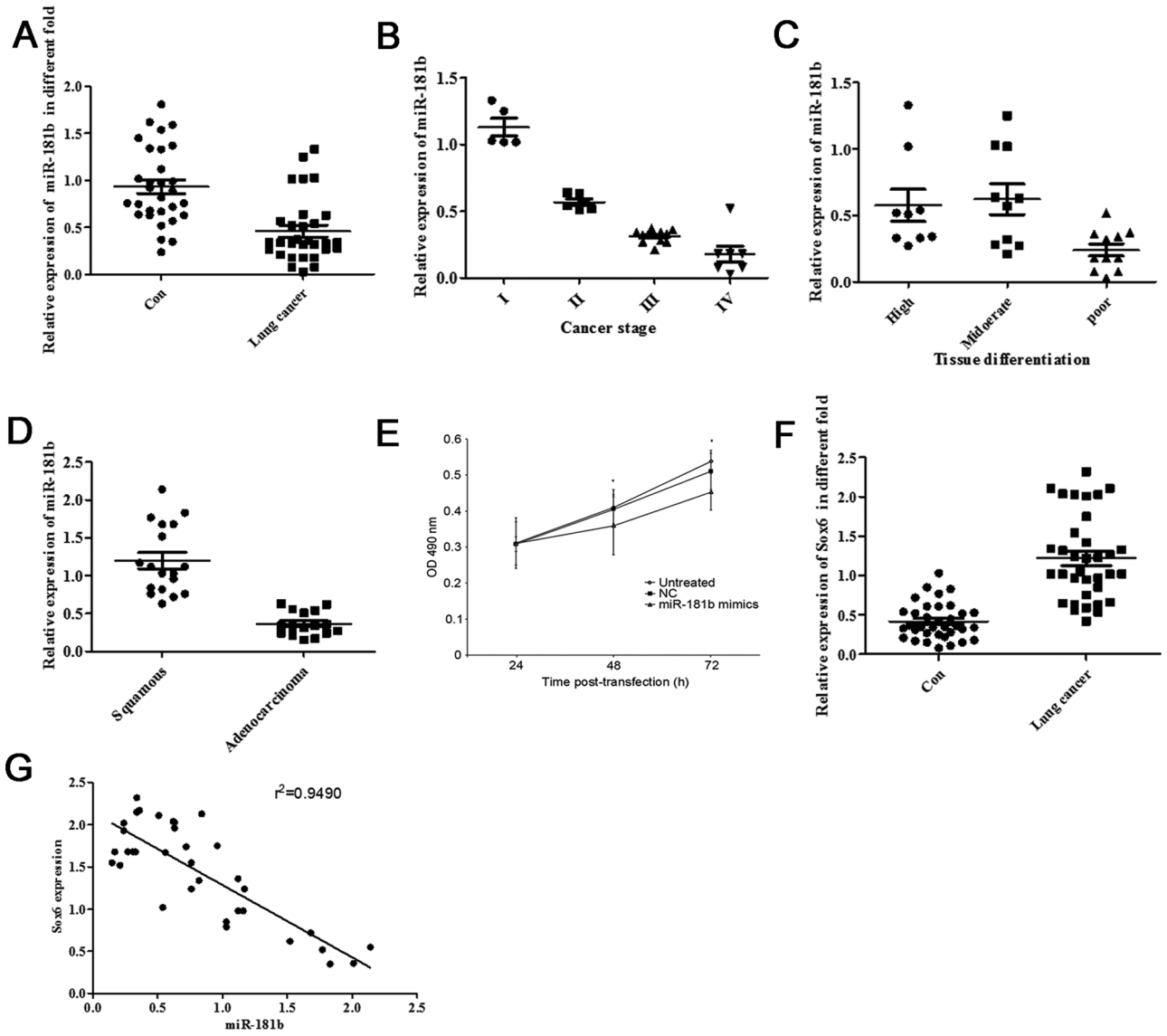

Fig. 1. Downregulation of miR-181b in lung cancer tissue specimens and cell proliferation was assessed using CCK-8 assay. Total RNA was isolated from matched normal and cancerous lung tissue using TRIzol. miR-181b expression was assessed using SYBR reverse transcription-quantitative polymerase chain reaction and normalized to U6 expression. (A) Quantification of miR-181b expression in matched lung cancer tissues and adjacent normal tissues. (B) Quantification of miR-181b expression in four different clinical stages of cancer. (C) Quantification of miR-181b expression in lung cancer tissues with poor, moderate and high differentiation. (D) Quantification of miR-181b expression in squamous cell carcinoma and adenocarcinoma. *P < 0.05, **P < 0.01. miR, microRNA. (E) CCK-8 assay was performed on A549 cells transfected with miR-181b mimic or NC and OD of each well was measured at the indicated time points with a microplate spectrophotometer at 490 nm. (F) RT-q PCR was measured So x 6 m RNA expression in lung cancer tissues and normal tissues. (G) Pearson's correlation analysis of miR-181b expression with Sox6 in 35 lung cancer tissues. *P < 0.05 vs. untreated group. CCK-8, cell counting kit-8; miR, microRNA; NC, negative control; OD, optical density.

Technology Co., Ltd.) Following with the manufacturer's instrucyion. All experiments were performed at least three times. Measurements of luminescence were performed using a luminometer (Glomax 20/20; Promega Corporation, Madison, WI, USA).

2.9. Statistical analysis

All data were presented as the mean ± standard deviation of three independent experiments. Results analysis was performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 Software (San, Diego, IL, USA). P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. miR-181b expression is suppressed in lung cancer tissues

The expression of miR-181b was examined in all 53 pairs of lung cancer and matched adjacent normal tissues. Expression levels of miR-181b were significantly downregulated in cancerous tissue samples compared with adjacent normal tissues (P < 0.05; Fig. 1A). To investigate the expression pattern of miR-181b in the development of lung cancer, the relevance between miR-181b expression and clinicopathological feature was also examined. miR-181b expression was significantly downregulated in later stages (II, III and IV) compared with stage I cancer (P < 0.05; Fig. 1B). miR-181b expression in poorly differentiated carcinoma tissues was significantly lower compared with highly or moderately differentiated carcinoma tissues (P < 0.05 ; Fig. 1C). There was no significant difference in miR-181b expression

observed between NSCLC and SCLC; however, squamous cell carcinoma exhibited a significant upregulation in miR-181b expression compared with adenocarcinoma ($P < 0.05$; Fig. 1D). The Sox6 was also upregulated in lung cancer tissues compared with normal tissues (Fig. 1F). The correlation between miR-181b and Sox6 expression was assessed that miR-181b was negatively correlated with Sox6 (Fig. 1G). These results suggest that miR-181b was significantly downregulated in lung cancer tissues compared with normal tissues, and its expression was associated with the degree of differentiation and clinical stages of lung cancer.

3.2. Overexpression of miR-181b inhibits A549 cell proliferation

To investigate the influence of miR-181b on phenotypes of lung cancer cells in vitro, A549 cells were transiently transfected with mature miR-181b mimic (miR-181b) or the NC. A cell counting kit-8 proliferation assay indicated that A549 cells transfected with miR-181b exhibited a significant increase in cell proliferation compared with untreated or NC-transfected cells ($P < 0.05$; Fig. 1E). These finding suggested that miR-181b may play a critical role in the development of lung cancer by inhibiting the proliferation of lung cancer cells.

3.3. miR-181b influences cell migration and invasion of lung cancer cell line

The expression of miR-181b was assessed in transfected A549 cells. The western blot results confirmed the expression of miR-181b was increased in A549 cells, compared with NC group (2 A). Following transfection, the effect of miR-181b on the migration and invasion of lung cancer cells was assessed. Transwell assays demonstrated that transfection with the miR-181b mimic significantly suppressed the number of A549 cells that migrated compared with NC transfection ($P < 0.05$; Fig. 2B). An invasion assay demonstrated that a similar reduction in cell migration was observed in cells transfected with miR-181b mimic compared with those transfected with NC ($P < 0.05$; Fig. 2C).

3.4. miR-181b disrupts the cell cycle of A549 cells

Following the induction of miR-181b overexpression in A549 cells, flow cytometry analysis was used to investigate the effect on the cell cycle. The rate of apoptosis was increased in the miR-181b mimics group when compared with NC group (Fig. 3A). Furthermore, the percentage of cells in G1 phase was significantly upregulated in the miR-181b group compared with the NC group ($P < 0.05$) and the proportion of cells in S phase was significantly decreased in the miR-181b group compared with the NC group ($P < 0.01$; Fig. 3B). Western blot analysis was also performed, and it was demonstrated that the expression of cell cycle-related proteins Cyclin D1 and Cyclin E1 markedly decreased with miR-181b overexpression (Fig. 3C). These results suggest that miR-181b may be able to initiate G1/S phase arrest via downregulating the expression of cell cycle-related proteins.

3.5. Sox6 3′-UTR is a target for miR-181b

To identify the target of miR-181b associated with lung cancer progression, putative target genes were searched with a focus on Sox6. Western blot analysis revealed that transfection of miR-181b markedly decreased the expression of Sox6 in A549 cells (Fig. 4A). The putative binding site in the 3′UTR of Sox6 was subsequently cloned into a luciferase reporter construct and used to measure the effects of the miR-181b mimic in HEK293 T cells. Luciferase activity was significantly lower in cells co-transfected with pMIR-REPORT-Sox6 3′-UTR and miR-181b compared with those co-transfected with pMIR-REPORT-Sox6 3′-UTR and NC (Fig. 4B). No significant differences in luciferase activity were observed between cells co-transfected with pMIR-REPORT-Sox6 3′-UTR mutant and miR-181b or NC. These findings suggest that Sox6 is a direct target of miR-181b.

3.6. Knockdown of Sox6 blocks the migration and invasion of A549 cells

To investigate whether miR-181b inhibits the development of lung cancer via the target gene Sox6, Sox6 expression was silenced (Fig. 6C).

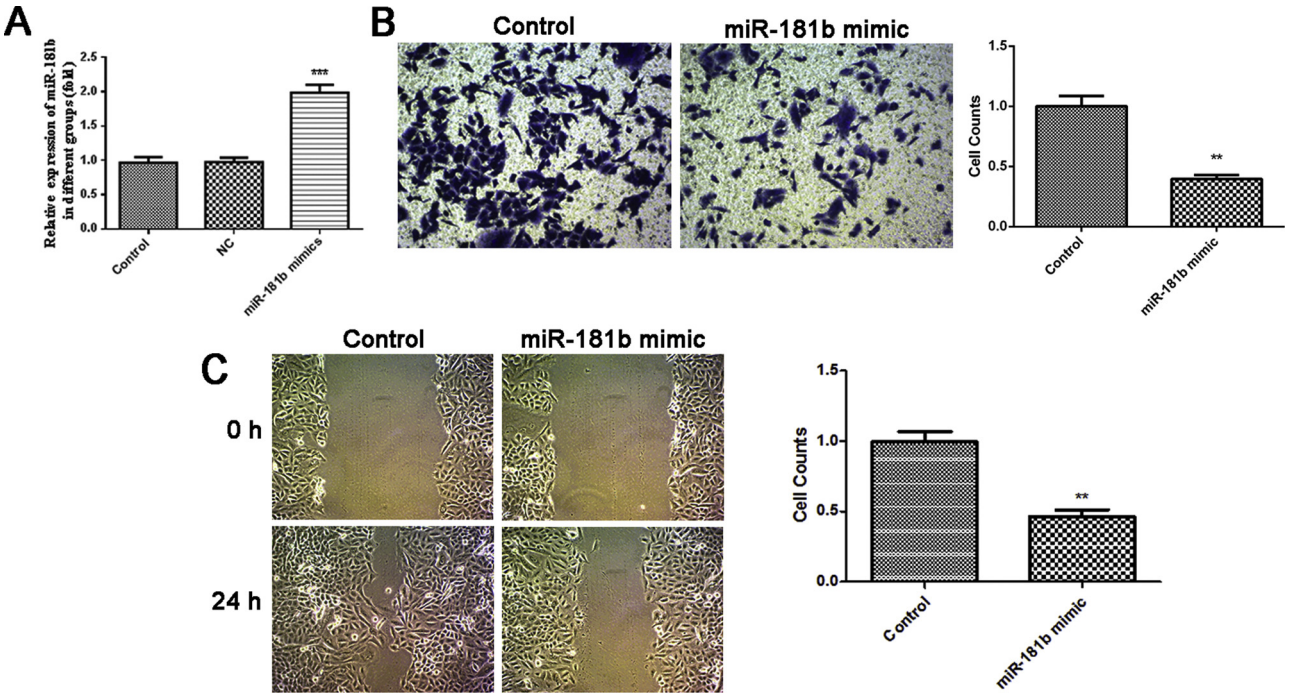

Fig. 2. miR-181b inhibits migration and invasion of A549 cells. (A)Western blot was performed and expression of miR-181b. Transwell (B) migration and (C) invasion assays of A549 cells transfected with miR-181b mimic or NC. Cells that migrated through the filter into the lower wells were quantified using Giemsa staining (magnification, ×200) and expressed as the total cell number in lower wells. *$P < 0.05$ vs. NC group. miR, microRNA; NC, negative control.

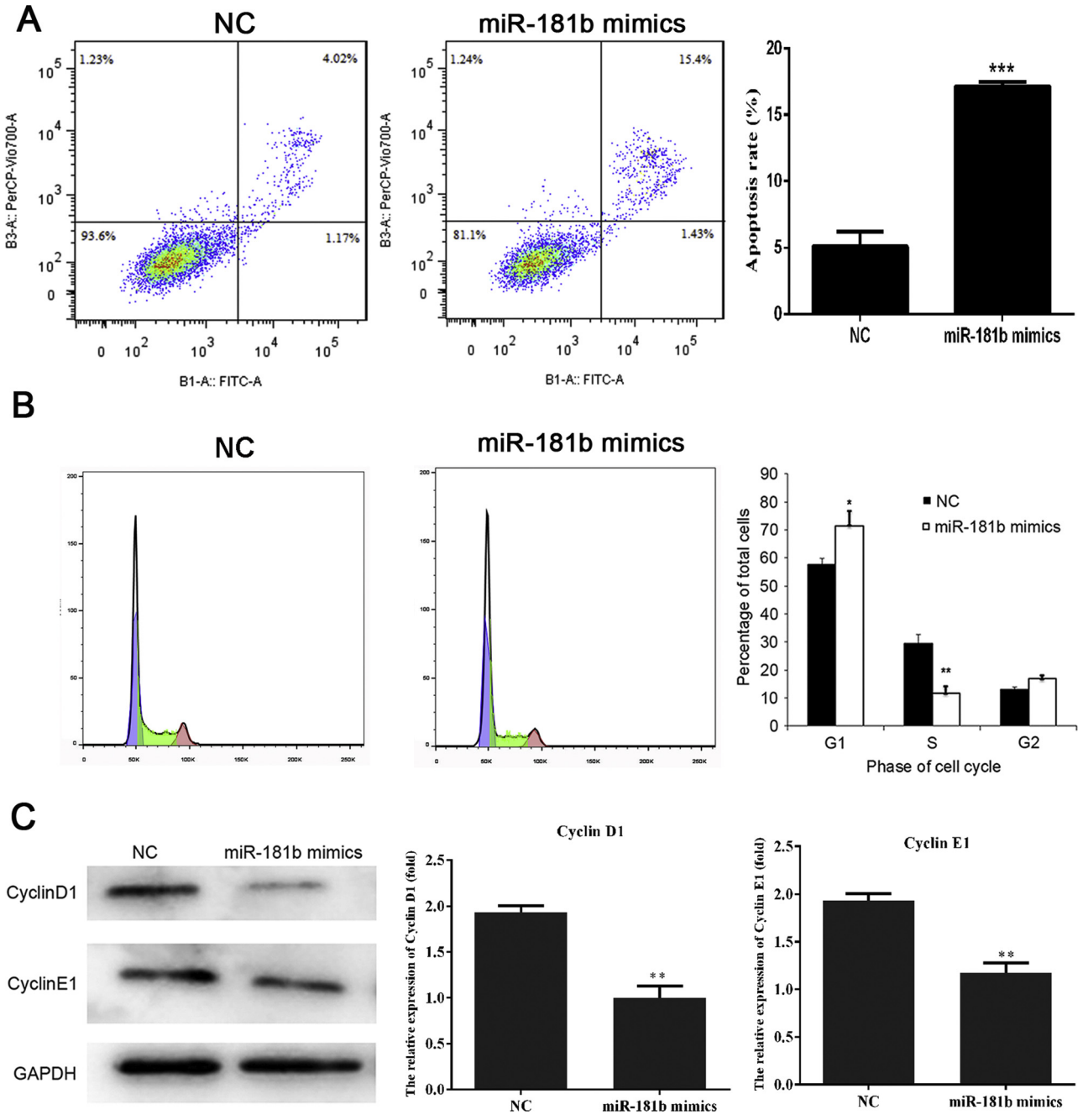

Fig. 3. miR-181b affects cell cycle distribution. (A) Cell apoptosis was analyzed by flow cytometry 24 h post-transfection of A549 cells with miR-181b mimic or NC (B) Cell cycle distribution was analyzed by flow cytometry 24 h post-transfection of A549 cells with miR-181b mimic or NC. (B) Western blot analysis of Cyclin D1 and Cyclin E1 expression with GAPDH as an internal control. *P < 0.05 vs. NC group, **P < 0.01 vs. NC group. miR, microRNA; NC, negative control.

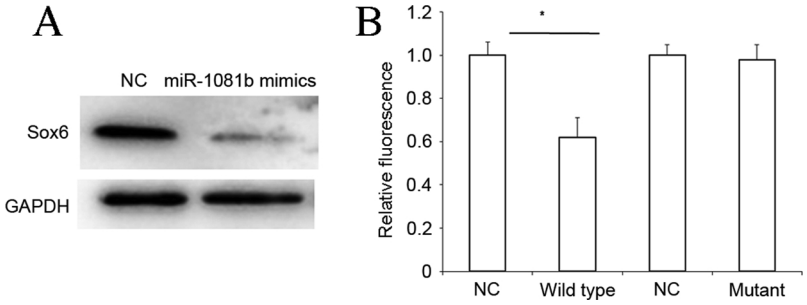

Fig. 4. Sox6 is a direct downstream target for miR-181b. (A) Western blot assays of Sox6 protein expression in A549 cells transfected with miR-181b mimic or negative control with GAPDH as an internal control. (B) Luciferase activity assays of luciferase vectors with wild-type or mutant Sox6-3′ untranslated region following co-transfection with miR-181b mimic or NC. The luciferase activity was normalized to pMIR-REPORT β-gal luciferase activity. *P < 0.05. Sox6, sex determining region Y-related high mobility group-box 6; miR, microRNA; NC, negative control.

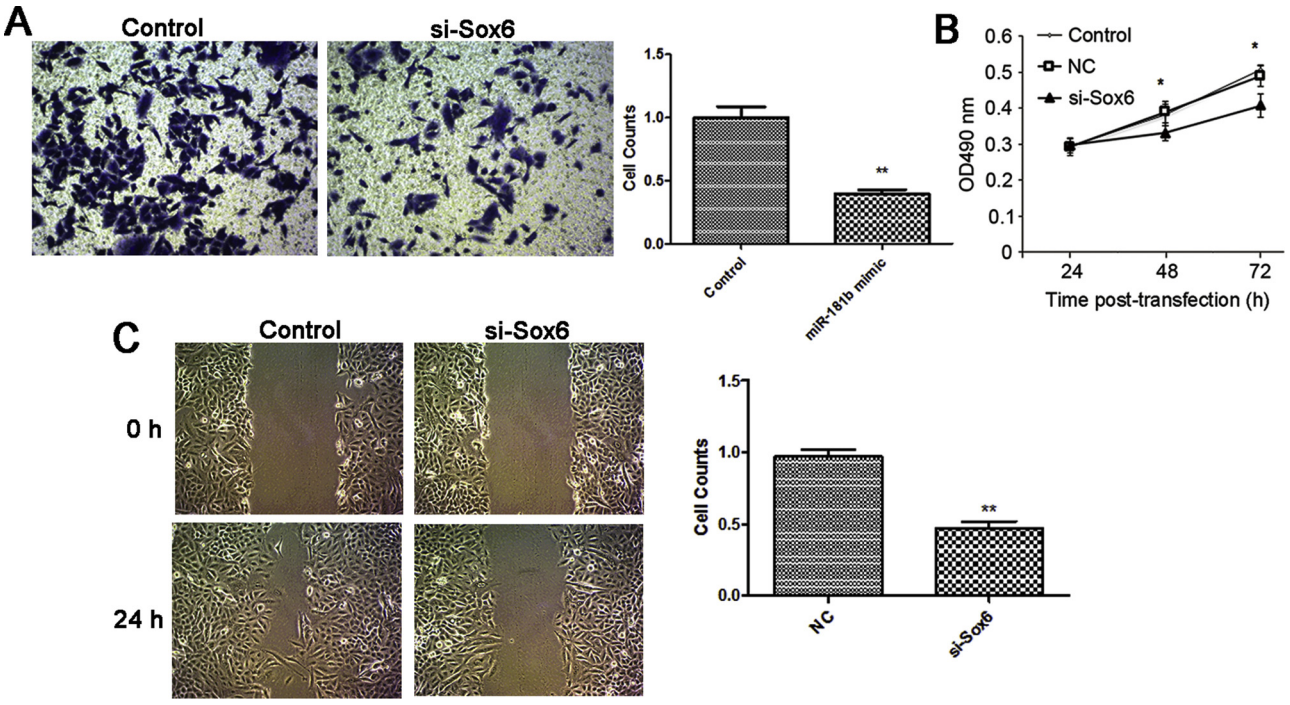

Fig. 5. Sox6 protein effects cell proliferation, migration and invasion. A549 cells were transfected with si-Sox6 or NC. (A) Transwell migration and invasion assays of A549 cells. Cells in the lower wells were stained and counted under a microscope (magnification ×200). (B) CCK8 assay was performed to monitor the proliferation of A549 cells post-transfection. *P < 0.05 vs. NC group, **P < 0.01 vs. NC group. Sox6, sex determining region Y-related high mobility group-box 6; si, small interfering RNA; CCK8, cell counting kit-8; NC, negative control.

Knockdown of Sox6 significantly suppressed the proliferation of A549 cells (P < 0.05; Fig. 5B) and a significant G1/S arrest was also observed in A549 cells transfected with si-Sox6 (P < 0.05; Fig. 6B). Western blot analysis revealed that cell cycle-related proteins Cyclins D1 and E1 were downregulated when Sox6 expression was silenced (Fig. 6C).

The quantified flow cytometry results demonstrated that a significantly greater number of cell apoptosis in si-Sox6 group (Fig.6A). The migration and invasion abilities of A549 cells transfected with si-Sox6 was assayed and demonstrated to be suppressed compared with NC transfected cells (P < 0.01 and P < 0.05, respectively; Fig. 5A). These findings suggest that suppression of Sox6 expression is able to inhibit the development of lung cancer, indicating that So x 6 may function as an oncogene.

4. Discussion

For ~80% of patients with lung cancer, invasion and distant metastasis occurs prior to first diagnosis, which is the primary reason for the typically poor prognosis of these patients [19]. It is therefore critical to inhibit the invasion and metastasis of lung cancer cells when attempting to reduce lung cancer mortality. Recent investigations have demonstrated that miRNA expression is dysregulated in patients with lung cancer, and it has been discovered that multiple miRNAs function as oncogenes or tumor suppressors that regulate the development of lung cancer. For example, the expression of miR-16 is reduced in A549 cells compared with normal bronchial epithelial cells, and overexpression of miR-16 is able to reduce A549 cell proliferation and promote apoptosis [20]. Additionally, the expression of miR-21, which regulates multiple mechanisms associated with cell proliferation, apoptosis and tumor invasion, is upregulated in lung cancer tissues and serves a crucial role in tumorigenesis and the progression of lung squamous cell carcinoma [21]. A number of novel cancer-related miRNA molecules have been discovered; it has been demonstrated that the miR-181 family, which is associated with drug resistance, tumor invasion, metastasis and apoptosis, is also associated with tumorigenesis [22]. However, the biological role and underlying mechanisms by which the miR-181 family is associated with lung cancer development remain to be elucidated.

In the previous study, it was demonstrated that miR-181b expression was remarkable downregulated in lung cancer tissues and that this downregulation was significantly greater in adenocarcinoma compared with squamous cell carcinoma. miR-181b expression in poorly or moderately differentiated carcinoma tissues was higher than in well-differentiated tissues, manifesting that miR-181b expression may be associated with the differentiation of lung cancer. Additionally, miR-181b expression was downregulated with increasing clinical stages of lung cancer. In vitro experiments demonstrated that miR-181b overexpression in A549 cells was induced by transfection of miR-181b mimic and this overexpression inhibited proliferation, migration and invasion. Furthermore, flow cytometry and western blot analysis indicated that miR-181b overexpression was able to disrupt the cell cycle of A549 cells via a G1/S block and downregulate the expression of cell cycle-related proteins, and the percentage of cells in the G0/G1 phase was increased in the miR-181b mimics group. In addition, miR-181b overexpression elevated cell apoptosis significantly.

The finding suggested that ectopic expression of miR-181b is associated with the development of multiple tumors and several miR-181b target genes have been identified. For instance, Zhi et al [23] stated that miR-181b was able to expedite the proliferation, metastasis and invasion of astrocytoma cells through targeting NOVA1. Lu et al [24] demonstrated that miR-181b was able to increase drug sensitivity in acute myeloid leukemia via targeting high mobility group box 1 and MCL1.

The present study demonstrated that miR-181b functions by targeting Sox6. Sox6 was predicted to be a target of miR-181b via bioinformatics, and confirmed by a dual luciferase report assay, which revealed that miR-181b was capable of directly bind to the 3′ UTR of So x 6 mRNA. Western blot analysis further supported that miR-181b was able to downregulate the expression of Sox6 protein. Sox6, a protein that binds to DNA through a high mobility group domain that is

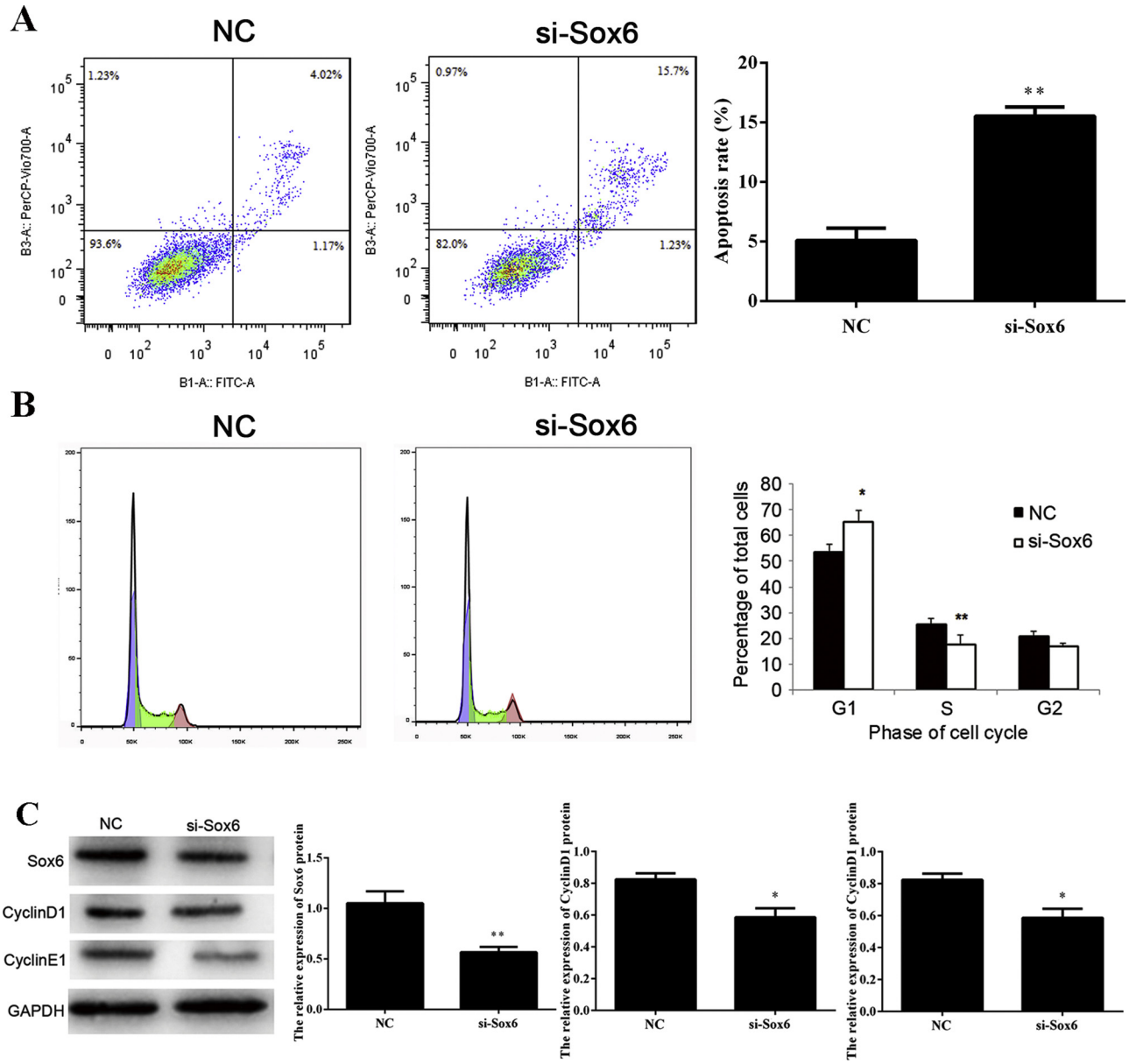

Fig. 6. Sox6 protein acts as an oncogene in lung cancer tissues in vitro. A549 cells were transfected with si-Sox6 or NC. (A) Cell apoptosis was analyzed by flow cytometry 24 h post-transfection of A549 cells with si-Sox6 or NC. (B) Flow cytometry analysis of cell cycle distribution at 24 h post-transfection. (C) Western blot analyses were used to determine Sox6, Cyclin D1 and Cyclin E1 protein expression with GAPDH as an internal control.*P < 0.05 vs. NC group, **P < 0.01 vs. NC group. Sox6, sex determining region Y-related high mobility group-box 6; si, small interfering RNA; NC, negative control.

highly conserved among species, belongs to the D subfamily of sex-determining region y-related transcription factors [25,26]. The expression of Sox6 is low in multiple. Sox6 is associated with tumorigenesis as it is a transcription factor that serves a crucial role in cell proliferation and differentiation, and has also previously been reported to regulate cardiac development and inflammation [27–29]. The Low expression of So x 6 may accelerate tumorigenesis and progression [30]. Li H suggested that miR 208 promote cell growth by down-regulating Sox6 level, in the human esophageal squamous cell carcinoma [31]. Moreover, miR-766 promotes human colorectal cancer cell proliferation via targeting Sox6 [32]. In the present study, Sox6 expression was silenced using siRNA to confirm that it is a target of miR-181b. CCK-8 and Transwell assays demonstrated that suppressed Sox6 expression inhibited proliferation, migration and invasion of A549 cells, similar to the effects observed with overexpression of miR-181b.

In conclusion, the results of the present study indicate that miR-181b may function as a tumor suppressor in patients with lung cancer by targeting Sox6, the downregulation of which promotes the metastasis of tumors. These findings may potentially have therapeutic applications for the treatment of lung cancer by inhibiting metastasis.

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Acknowledgements

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