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Original Article

MicroRNA-193b modulates proliferation, migration, and invasion of non-small cell lung cancer cells

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MicroRNAs have been reported to be closely related to the development of human lung cancers. However, the functions of microRNAs in non-small cell lung cancer (NSCLC) remain largely undefined. Here, we investigated the role of microRNA-193b (miR-193b) in NSCLC. Our data showed that miR-193b was markedly down-regulated in NSCLC cancer tissues compared with adjacent normal tissues. The NSCLC cell line (A549) transfected with the miR-193b exhibited significantly decreased proliferation, migration, and invasion capacities when compared with the control cells. In contrast, inhibition of miR-193b increased the proliferation, migration, and invasion of A549 cells. Moreover, miR-193b repressed the expressions of cyclin D1 and urokinase-type plasminogen activator in A549 cells. These data suggest that miR-193b is a tumor suppressor in NSCLC.

Keywords microRNA-193b; NSCLC; proliferation; migration; invasion

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Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death in male individuals, and the fourth most commonly diagnosed cancer and the second leading cause of cancer death among females [1]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for 80% of lung cancer cases [1,2]. Despite intensive research and resources being dedicated to elucidate the molecular mechanisms of NSCLC, the precise processes of initiation and progression remain unclear.

MicroRNAs (miRNA) are non-coding RNAs (20–24 nucleotides) that regulate gene expression post-transcriptionally by targeting mRNAs for translational repression or cleavage [3]. Recent studies have shown that miRNAs are dramatically down-regulated in a variety of human cancers, including NSCLC. The results indicated that miRNAs play a key role in the processes of cancer initiation, progression, and metastasis [4,5]. MicroRNA-193b (miR-193b), a putative tumor suppressor, is down-regulated in a variety of cancers, and regulates cancer cell proliferation, migration, invasion, and metastasis [6–10]. However, the role of miR-193b in NSCLC is not clear.

In this study, we investigated the expression of miR-193b in NSCLC tissue and the biological functions of miR-193b in NSCLC cell line A549. Our data revealed that miR-193b could regulate the behaviors of A549 cells including cell growth, migration, and invasion.

Materials and Methods

Tissue samples

The NSCLC tissue specimens and adjacent lung tissue specimens were obtained from 20 patients at the First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China) after surgical resection. The detailed information of the 20 patients is listed in **Supplementary Table S1**. The tumor tissues and the adjacent normal tissues were frozen in liquid nitrogen after resection. The study was performed with the approval of the Medical Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from lung tissues using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's manual. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays were performed using the TaqMan miRNA assay (Applied Biosystems, Foster City, USA) according to the

manufacturer's instructions. In brief, 10 ng of RNA was reversed transcribed using the universal primer. After that 1.33 μl of RT product was mixed with universal PCR master mix and Taqman MicroRNA Assay mix, and applied to PCR reaction. PCR was carried out using the following parameters: activation at 95°C for 10 min, denatured at 95°C for 15 s, and extended at 60°C for 60 s for 40 cycles. The expression level of miR-193b was normalized to the expression level of U6 small nuclear RNA.

Cell culture

NSCLC cell line A549 was obtained from American Type Culture Collection (ATCC, Manassas, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, USA) and penicillin–streptomycin mixed solution. Cells were maintained at 37°C in an atmosphere containing 5% CO_2 and 100% humidity.

Transfections

A549 cells were transiently transfected with 10 nM of the chemically synthesized miR-193b (sense sequence: 5′-AACUGGCCCUCAAAGUCCCGCU-3′), negative control miRNA (NC, sense sequence: 5′-UUCUCCGAACGUGUCACGU-3′), miR-193b inhibitor (miR-193bi, sequence: 5′-AGCGGGACUUUGAGGGCCAGUU-3′), or negative control inhibitor (NCi, sequence: 5′-CAGUACUUUUGUGUAGUACAA-3′) (Genepharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After 24-h transfection, cells were used for subsequent experiments including proliferation, migration, and invasion assays.

Cell proliferation assay and cell cycle analysis

Cells were transfected with miR-193b, NC, miR-193bi, or NCi. After 24 h, cells were detached and re-plated in 6-well plates at 2×10^5 cells per well. The whole cell number was counted directly at the indicated time.

For cell cycle analysis, cells were fixed, stained with propidium iodide and examined with a fluorescence-activated cell sorting flow cytometer (Beckman Coulter, Pasadena, USA), and DNA histograms were analyzed using 'Multicycle' software.

Wound healing assay

Cells transfected with miR-193b, NC, miR-193bi, or NCi were plated in 35-mm dishes. When cells grew to confluence, a line was traced with a pipette tip. Then A549 cells were washed with serum-free medium and incubated with DMEM. The wound was photographed at 0 and 24 h.

Cell invasion assay

The invasion of A549 cells was measured with Matrigel-coated transwell chambers (Millipore, Billerica, USA). Transwell inserts with 8-μm pores were coated with Matrigel (Invitrogen). A549 cells transfected with miR-193b, NC, miR-193bi, or NCi were seeded into the upper chambers in DMEM supplemented with 10% FBS. The same medium was also placed in the lower wells. After 24 h, cells that invaded to the lower surface of the transwell membrane were fixed with 95% ethanol and stained with 0.2% crystal violet solution for 30 min. Pictures were taken under a wild-field microscope. Crystal violet was dissolved in 10% acetic acid and the absorption at 590 nm was measured. The absorption of control group was normalized to 1 and other groups were normalized to the control group.

Western blot analysis

Cells or tissues were lysed with RIPA lysis buffer (100 mM Tris at pH 8.0, 1% Triton X-100, 100 mM NaCl, 0.5 mM EDTA), and the lysate was cleared by centrifugation at 13,200 *g* for 30 min. Protein amount was measured with Bradford method. Then 15 μg of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Whatman nitrocellulose membrane (GE Healthcare, Life Sciences, Piscataway, USA). Membrane was blocked with 3% bovine serum albumin in Tris-Buffered Saline Tween-20 (TBST) buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), followed by incubating with anti-cyclin D1 antibody (CCND1, 1:1000, Santa Cruz Biotechnology, Santa Cruz, USA), anti-urokinase-type plasminogen activator (uPA, 1:1000, Santa Cruz Biotechnology), or anti-α-tubulin (1:1000, Santa Cruz Biotechnology). After washing with TBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and detected with the SuperSignal protein detection kit (Thermo Fisher Scientific).

Target gene search and plasmids construction

To identify potential target genes of miR-193b, the TargetScan (http://www.targetscan.org), MIRanda (http://www.microrna.org), and PicTar (http://pictar.mdc-berlin.de) were used. The potential targets including *cyclin D1, PHD finger protein 15, mitogen-activated protein kinase kinase kinase 3, bromodomain* and *PHD finger containing1, urokinase-type plasminogen activator,* in all the databases were selected.

Wild-type 3′-untranslated regions (3′-UTRs) of *cyclin D1* (*CCND1*) gene containing predicted miR-193b target sites were amplified by PCR from A549 cDNAs. Seed sequence-deleted mutant of *CCND1* 3′-UTR was generated by overlap-extension PCR method. During the first PCR

cycle, the 5′ fragment and 3′ fragment of *CCND1* 3′-UTR was amplified using the primer sets CCND1-F/CCND1-mut-R and CCND1-mut-F/CCND1-R. The PCR products were mixed and used as the template, and the second PCR cycle was carried out using the primer set CCND1-F/CCND1-R. Both the wild-type and mutated 3′-UTR fragments were cloned into the downstream of firefly luciferase coding region between the *Eco*RV and *Not*I sites of the modified pGL3-control plasmid (Promega, Madison, USA).

The wild-type and deletion mutant of *uPA* 3′-UTR were generated using the same strategy and cloned between *Xba*I and *Nde*I sites of the modified pGL3-control plasmid. All the primers are listed in **Supplementary Table S2**.

Luciferase assays

A549 cells in 24-well plates were transfected with 100 ng firefly luciferase reporter plasmid, 10 ng pRL-TK plasmid (Promega), together with 10-pmol mir-193b or NC. Cells were harvested 48 h after transfection, and dual luciferase activity was measured with dual luciferase assay kit (Promega) and normalized to renilla signals.

Statistical analysis

The expression of MiR-193b in tissue samples were analyzed using the Mann–Whitney *U* test for comparing the results of two groups. Other data were expressed as the mean ± SD and evaluated with a double-sided Student's *t* test. Values of $P < 0.05$ were accepted as statistically significant.

Results

miR-193b is down-regulated in NSCLC cancer tissues compared with in normal lung tissues

To test whether miR-193b was correlated with NSCLC development, we measured the expressions of miR-193b in independent series of NSCLC tumors and adjacent normal lung tissues using quantitative RT-PCR. The results showed that the expressions of miR-193b in cancer tissues were significantly lower than those in normal lung tissues (**Fig. 1**, $P < 0.01$, Mann–Whitney), suggesting that the down-regulation of miR-193b might be related with human NSCLC development.

miR-193b inhibits cell proliferation and induces G1 phase arrest in A549 cells

To test whether miR-193b affected the behavior of NSCLC cell, we select A549 cell line for further analysis. First, we detected the proliferation rates of A549 cells with miR-193b or miR-193bi transfection. Compared with the cells transfected with negative control miRNA, the proliferation rate of the cells transfected with miR-193b was significantly decreased [**Fig. 2(A)**]. In contrast, the expression

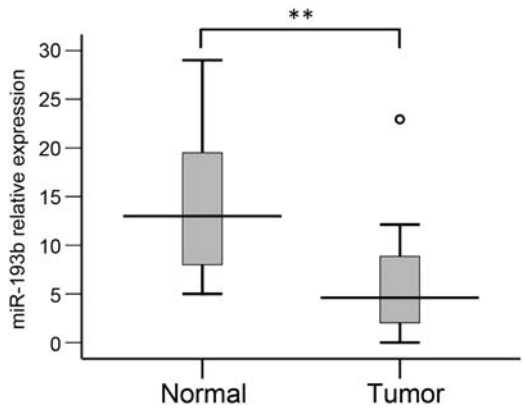

Figure 1 The expression profile of miR-193b in human NSCLC The expression levels of miR-193b in 20 NSCLC cancer tissues and adjacent normal tissues were assessed by quantitative RT-PCR, which was normalized to U6 small nuclear RNA (**$P < 0.01$, Mann–Whitney *U* test).

of miR-193bi resulted in significantly enhanced proliferation [**Fig. 2(B)**], indicating that miR-193b may inhibit A549 cell proliferation.

Altered cell proliferation might be caused by cell cycle changes. Therefore, we detected cell cycle distribution by flow cytometry. Data showed that A549 cells transfected with miR-193b had increased numbers of cells in G0/G1 and decreased numbers of cells in S and G2/M [**Fig. 2(C)**]. Accordingly, miR-193b inhibitor transfection decreased cell numbers in the G0/G1 phase and increased cell numbers in the S and G2/M phase [**Fig. 2(D)**]. These data suggested that miR-193b triggers G1 arrest in A549 cell line.

miR-193b suppresses the migration and invasion of A549 cells

We further investigated the effects of miR-193b on A549 cell migration and invasion, two essential steps for malignant progression and metastasis. A549 cells transfected with the miR-193b, miR-193bi, NC, or NCi were applied to wound healing assays. The results showed that miR-193b significantly decreased the migration of A549 cells, whereas miR-193bi increased the migration of A549 cells (**Fig. 3**).

Matrigel invasion assays showed that A549 cells transfected with miR-193b displayed a remarkable decrease compared with those transfected with NC cells (0.53 fold, $P < 0.01$) [**Fig. 4(A,B,E)**], whereas cells transfected with miR-193bi significantly increased the invasion ability (1.38 fold, $P < 0.01$) [**Fig. 4(C,D,E)**]. These results suggested that miR-193b can promote A549 cell motility.

miR-193b represses the expressions of CCND1 and urokinase-type plasminogen activator (uPA) in A549 cells

Using bioinformatics analysis, we found that a series of 3′UTR regions of human genes contained potential

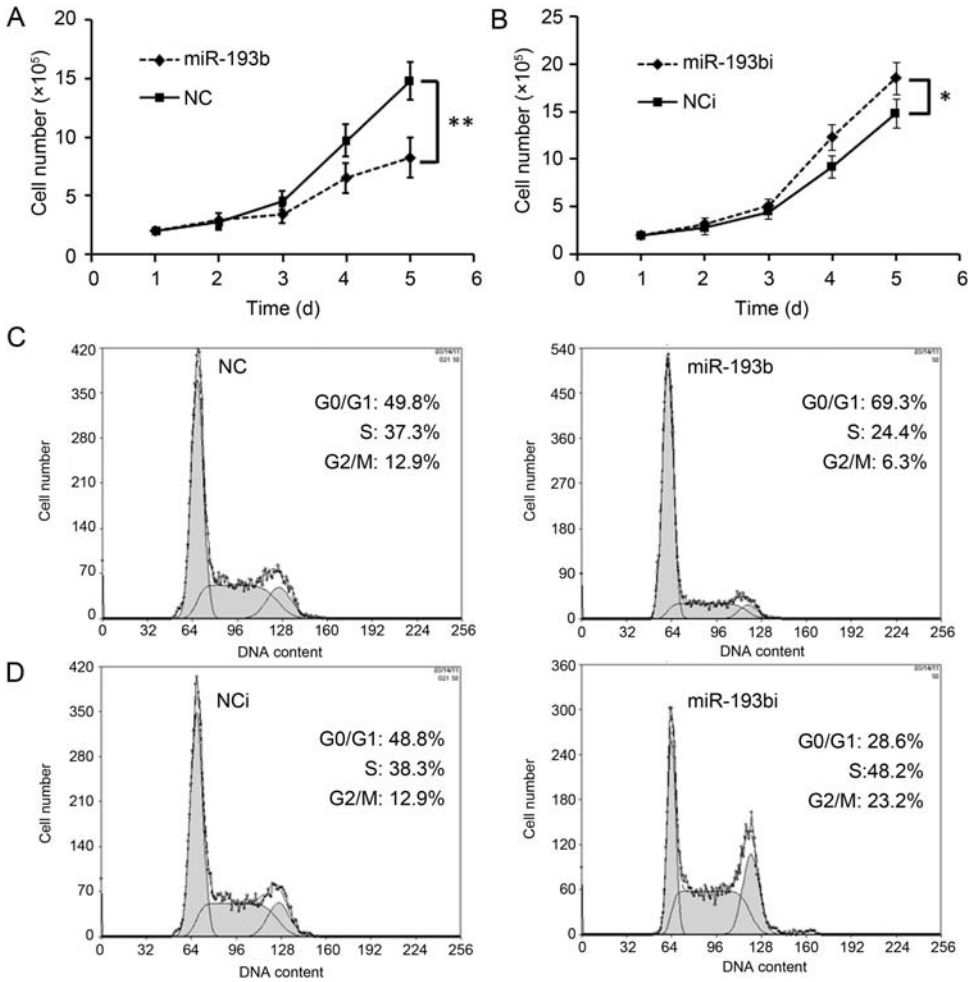

Figure 2 miR-193b inhibits proliferation of A549 cells A549 cells were transfected with the miR-193b (A) or inhibitor (B), and their cell proliferation rates were assessed by direct cell counting at indicated time. Mean ± SD of triplicate measurements. $*P < 0.05$; $**P < 0.01$. Cell cycle distributions of A549 cells transfected with the miR-193b (C) or inhibitor (D) were analyzed by fluorescence-activated cell sorting.

miR-193b-binding sequences. Among them, *CCND1* [10] and *uPA* [11] have been reported to be regulated in other cell lines. To determine whether *CCND1* and *uPA* are miR-193b target genes in NSCLC cells, we transfected A549 cells with miR-193b, miR-193bi, NC ,or NCi, and detected the expressions of CCND1 and uPA. Data showed that the protein levels of both CCND1 and uPA significantly decreased in miR-193b transfected cells, and increased in miR-193bi transfected cells [**Fig. 5(A)**]. These data demonstrated that miR-193b could inhibit the expressions of CCND1 and uPA in A549 cells. We have detected the decreased expressions of miR-193b in NSCLC. Therefore, we speculated that the expression levels of CCND1 and uPA should increase in these NSCLC tissues. Three cases from the 20 tissue samples were selected for the immunoblot analysis. Data showed that the expression levels of CCND1 and uPA in cancer tissues were higher than that in the adjacent normal tissues [**Fig. 5(B)**].

Luciferase assays were also carried out to confirm the regulatory roles of miR-193b in the expressions of *CCND1* and *uPA*. The 3′-UTRs of *CCND1* and *uPA* genes were cloned into the downstream of the coding sequence of luciferase. These constructs were co-transfected into A549 cells with miR-193b or NC. Data showed that miR-193b but not NC specifically decreased the luciferase levels from each reporter [**Fig. 5(C,D)**]. The inhibitory effects of miR-193b were abolished upon the deletion of the miR-193b-binding site within the 3′UTRs of *CCND1* and *uPA* [**Fig. 5(C,D)**]. These results suggested that miR-193b regulates the expressions of *CCND1* and *uPA* directly.

Discussion

In this study, we provided a line of evidence to demonstrate an inhibitory role of miR-193b in the development of NSCLC. Our data showed that miR-193b expression decreased in NSCLC tissues compared with in adjacent normal tissues. Overexpression of miR-193b decreased the proliferation, migration, and invasion of A549 cells, whereas expression of its inhibitor increased the

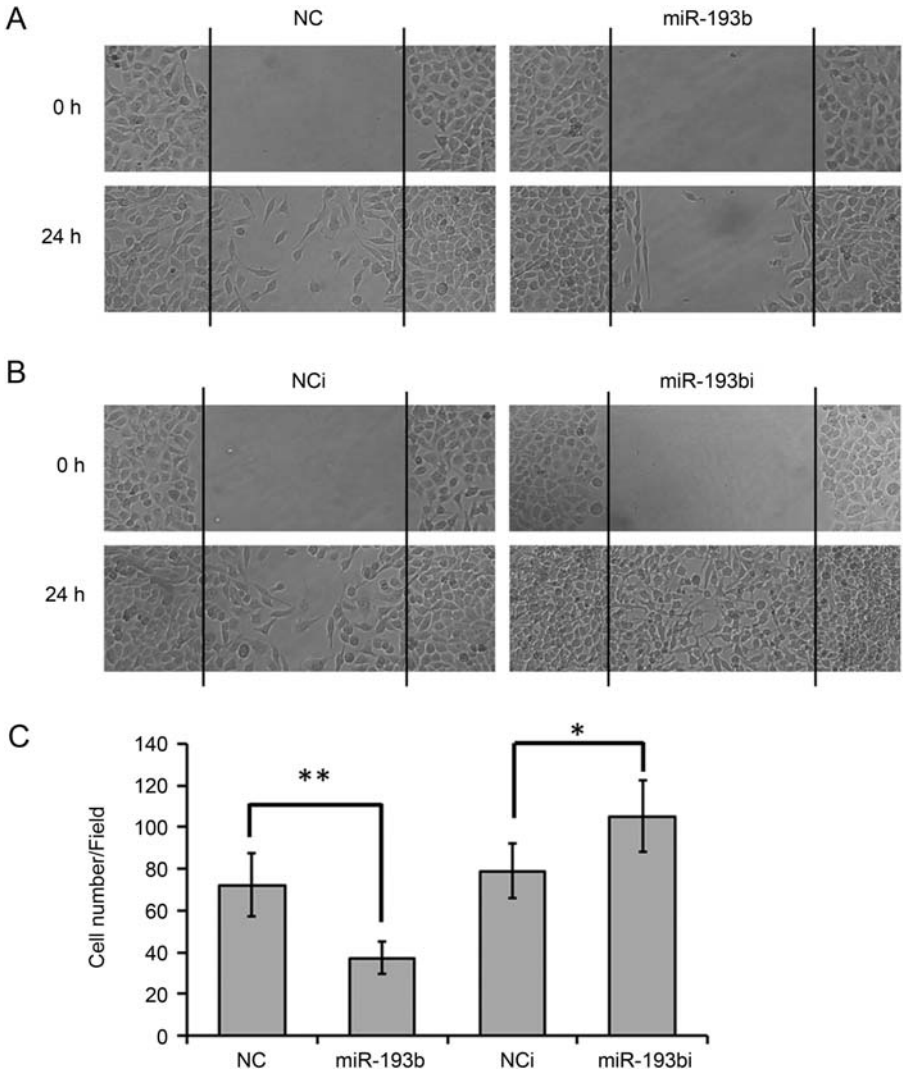

Figure 3 miR-193b suppresses the migration and invasion of A549 cells A549 cells transfected with miR-193b (A) or inhibitor. (B) Cells were subjected to wound healing assays and images were taken at 0 and 24 h. (C) Cells that migrated into the wound areas were counted and analyzed. Data shown are mean ± SD of triplicate measurements. $*P < 0.05$; $**P < 0.01$.

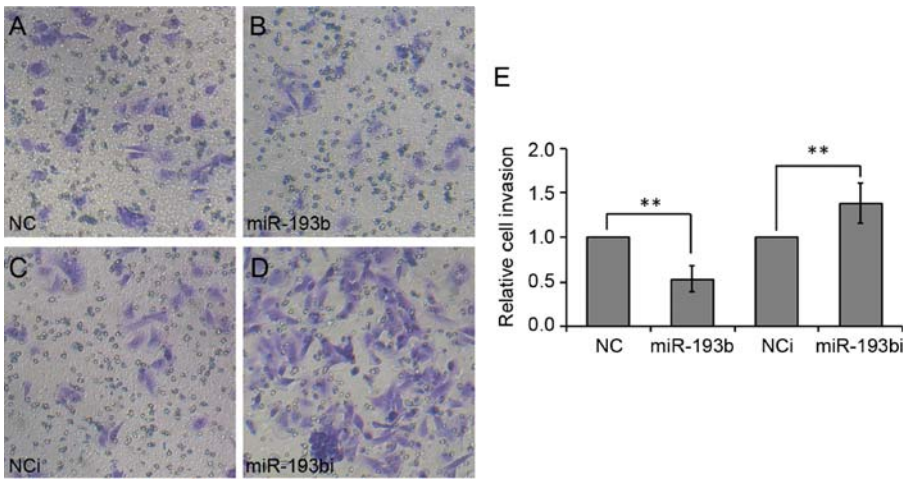

Figure 4 miR-193b suppresses the invasion of A549 cells A549 cells transfected with the miR-193b or inhibitor were subjected to matrigel migration assays. The migrated cells were stained with crystal violet for 30 min. Pictures were taken (A–D) and the absorptions at 590 nm were detected (E). Mean ± SD of triplicate measurements. $**P < 0.01$.

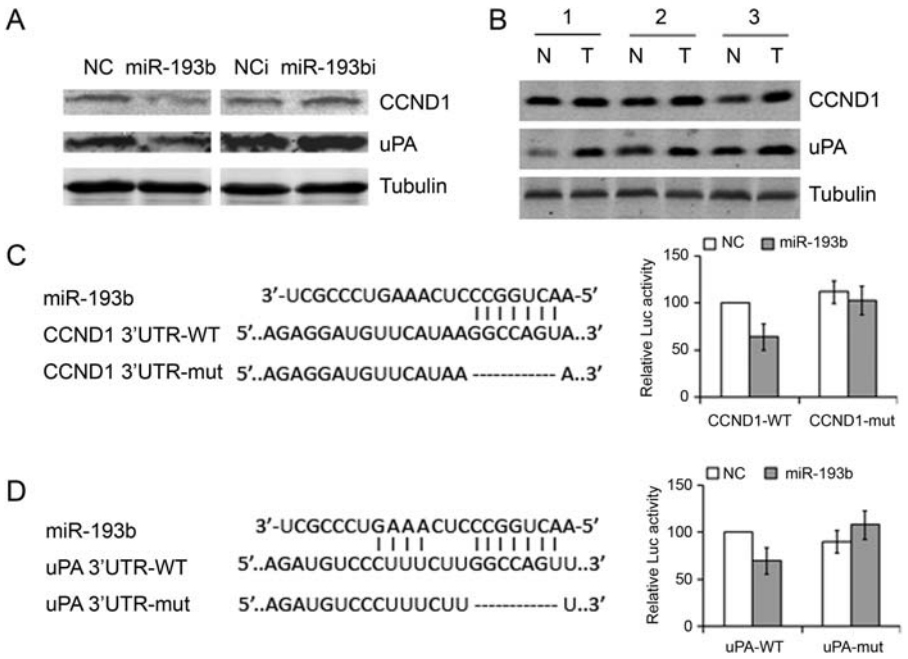

Figure 5 miR-193b suppresses the expressions of CNND1 and uPA (A) A549 cells were transfected with the miR-193b or inhibitor. The cell lysates were blotted with anti-CCND1, anti-uPA, and anti-tubulin. (B) The protein levels of CCND1 and uPA in three NSCLC cancer tissues (T) and adjacent normal tissues (N) were detected by immunoblot. (C) A549 cells were transfected with CCND1-UTR-Luc (CCND1-WT) or seed sequence-deleted mutant (CCND1-Mut), together with miR-193b. Luciferase activities were detected 24 h later. (D) A549 cells were transfected with uPA-UTR-Luc (uPA-WT) or seed sequence-deleted mutant (uPA-Mut), together with miR-193b. Luciferase activities were detected 24 h later.

proliferation, migration, and invasion of A549 cells. The progression of NSCLC is a complex process including initiation, promotion, and progression. Initial steps involve the disruption of a set of interdependent pathways controlling the homeostasis between cell growth and apoptosis. At later stages, cells may acquire invasive and metastatic properties. Our data implied that miR-193b influences many processes in NSCLC including both tumor growth and metastasis.

Abnormal cell cycle control is not only a critical step in NSCLC maintenance but also appears to be a critical early event. Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine a cell's progress through the cell cycle [12]. CCND1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, and therefore controls cell cycle G1/S transition. Mutations and overexpression of this gene, which alter cell cycle progression, are observed in a variety of tumors and may contribute to tumorigenesis [13,14]. Here, we found that miR-193b inhibited cell proliferation and induced G1 arrest in A549 cells (**Fig. 2**). Immunoblot and luciferase assays showed that miR-193b inhibited CCND1 expressions in A549 cells [**Fig. 5(A,C)**]. Accordingly, we observed the increased expressions of CCND1 in three NSCLC tissues [**Fig. 5(B)**]. These data indicated that miR-193b might regulate cell cycle G1/S transition by down-regulating CCND1 expression and therefore inhibit cell proliferation in A549 cells. Consistent with our data, miR-193b was reported to repress cell proliferation and regulate CCND1 expression in melanoma cells [10] and hepatocellular carcinoma cells [9].

The uPA is a serine protease, which catalyzes the conversion of the inactive zymogen plasminogen into the active broad-spectrum plasmin. Plasmin degrades matrix proteins and also activates other proteases, including some matrix metalloproteinases [15]. Through degrading the extracellular matrix proteins, uPAs regulate a serious of cancer progression processes including cancer cells motility, extracellular matrix invasion, and metastasis [16]. Overexpression of uPA has been reported to be associated with tumor growth, invasion, and metastasis [16–18]. It was reported that miR-193b significantly inhibited breast cancer cell invasion, and the growth and dissemination of xenograft tumors by regulating the expression of uPA [11]. NSCLC can metastasize to other organs such as bone or the liver. Our data showed that uPA is also a target of miR-193b in A549 cells (**Fig. 5**). As high expression level of uPA is related to cancer metastasis, miR-193b may function as a suppressor in the process of NSCLC metastasis by targeting uPA expression. Further studies are needed to fully elucidate the role of miR-193b in lung cancer metastasis by using mouse metastasis models such as tail vein injection models.

In conclusion, we found that miR-193b was down-regulated in NSCLC cancer, and that miR-193b inhibited NSCLC cancer cell proliferation and invasion *in vitro*.

These results indicate a suppressive role of miR-193b in the development of NSCLC, and implicate its potential application in cancer therapy.

Supplementary Data

Supplementary data are available at *ABBS* online.

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