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MicroRNA-16 inhibits the proliferation, migration and invasion of non-small cell lung carcinoma cells by down-regulating matrix metalloproteinase-19 expression

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Abstract. – OBJECTIVE: This study aims to investigate the expression of microRNA (miR)-16 in non-small cell lung carcinoma (NSCLC) and to identify its potential mechanism.

PATIENTS AND METHODS: A total of 45 NSCLC patients were included in the present work. NSCLC tissues and adjacent normal tissues were resected and collected. The Reverse Transcription-quantitative Polymerase Chain Reaction was used to determine miR-16 expression. Regulatory effects of miR-16 on proliferation, migration and invasion, and cell cycle of A549 cells were determined by Cell-Counting Kit 8 assay, transwell assay, and flow cytometry, respectively. Western blotting was performed to measure the protein expression of matrix metalloproteinase (MMP)-19 in cells overexpressing miR-16. Dual-luciferase reporter gene assay was conducted to identify the interaction between miR-16 and MMP-19.

RESULTS: MiR-16 expression in NSCLC significantly decreased compared with that in healthy tissue ($p<0.05$). The expression level of miR-16 was negatively correlated to the clinical staging of NSCLC. In addition, the expression of miR-16 in NSCLC patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis ($p<0.05$). *In vitro* studies demonstrated that miR-16 inhibited the proliferation, migration, and invasion of A549 cells. Western blotting analyses indicated that overexpression of miR-16 down-regulated the expression of MMP-19. Additionally, the dual-luciferase reporter gene assay determined that miR-16 directly regulated the expression of MMP-16.

CONCLUSIONS: The present study demonstrates that miR-16 acts as a tumor-suppressor gene by inhibiting the proliferation, migration, and invasion of NSCLC cells via downregulating MMP-19 expression.

Key Words:
MicroRNA-16, Non-small cell lung carcinoma, Matrix metalloproteinase-19.

Introduction

Lung cancer has the highest mortality and morbidity rates of all malignant tumors in the world, which poses a public health threat[1,2]. According to the clinical pathological classification, primary lung cancer may be divided into small cell lung carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC accounts for more than 85% of all types of lung cancer and encompasses three histological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell lung cancer[3-5]. The incidence rate of lung cancer in China is the highest of all tumors (25-33%) and increases annually[6]. Although numerous studies investigating NSCLC have been performed, the molecular mechanisms responsible for the onset and development of NSCLC remain unclear[7,8].

MicroRNA are a class of endogenous, highly conserved non-coding small-molecule RNAs that are 18-23 nucleotides long. MicroRNA binds to the 3′-untranslated region (UTR) of target gene mRNA and exerts its biological function by regulating the translation of mRNA[9]. It has been showed[10,11] that microRNA is widely involved in the incidence and development of tumors and serves as an oncogene and tumor-suppressor gene. Hong et al[12] demonstrated that miR-519a inhibits the incidence and development of gliomas by targeting the signal transducer and activator of transcription 3 signaling pathway. In

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addition, miR-101 downregulates the expression of zinc finger X-chromosomal protein in gallbladder carcinoma. It inhibits the proliferation, invasion, and metastasis of tumor cells by regulating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase and Smad signaling pathways[13]. Xu et al[14] demonstrated that miR-148a is able to inhibit the invasion and metastasis of breast cancer cells, which is associated with the prognosis of triple-negative breast cancer. We believed that microRNAs serve important biological functions in tumor cells. MiR-16 is a tumor-related small RNA molecule that is abnormally expressed in colon cancer, ovarian cancer, and gliomas, which is closely associated with the proliferation, invasion, and metastasis of tumor cells[15]. There have been few researches investigating the effect of miR-16 on NSCLC and the molecular mechanism of miR-16 in NSCLC remains unclear. The present study investigated the expression of miR-16 in NSCLC tissues and cell lines, as well as its mechanism. Our results may provide an experimental basis for understanding the molecular mechanism of NSCLC.

Patients and Methods

Patients

A total of 45 NSCLC patients (age range, 23-69 years; mean age, 43.5±1.5 years; 26 males and 19 females) undergoing surgical resection at the Affiliated Hospital of Jining Medical University (Jining, China) between September 2013 and December 2014 were enrolled in the present study. Prior to surgery, none of the patients received adjuvant therapy. The inclusion criterion was a diagnosis of NSCLC, and the exclusion criterion was the necessity of postoperative chemotherapy. NSCLC tissues and adjacent normal tissues were resected and collected. Among the 45 patients, 28 exhibited lymph node metastasis, which was determined by imaging and pathological examination of peritumoral lymph nodes.

Tumor-node-metastasis (TNM) staging followed the 2003 edition of American Joint Committee on Cancer (AJCC) breast cancer TNM staging standards[16,17]. Among the 45 patients, 13 were classified as stage I, 21 were stage II, and 11 were stage III. All the procedures were approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University. The written informed consent was obtained from all patients or their families.

Cells

Lung cancer cell line A549 was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). A549 cells were defrosted at 37°C and cultured in 10 mL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C and 5% CO_2 for 24 h. Following the cell culture, 5 mL of fresh high-glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) was replaced. The medium was replenished every two days and cells were passaged when they reached a confluence of 90%.

A549 cells were divided into a miR-negative control (miR-NC) group and miR-16 mimic group. When cell confluence had reached 70-90%, 1.25 μL of miR-NC or miR-16 mimics (20 pmol/μL; Guangzhou RiboBio, Co., Ltd., Guangzhou, China) and 1 μL of Lipofectamine® 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added to two individual vials each containing 50 μL Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 5 min later, the liquids in the two vials were mixed before standing still for 15 min. Subsequently, the mixture was added to each well for 6 h incubation at 37°C. The medium was changed to DMEM supplemented with 10% FBS and cells were cultured at 37˚C for 48 h under normal conditions prior to use.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Tumor tissues or matched adjacent normal tissues from patients (100 mg) were lysed in 1 mL of TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and ground into powder using liquid nitrogen. Total RNA was extracted using the phenol-chloroform method[18]. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Complementary deoxyribose nucleic acid (cDNA) was obtained by conducting reverse transcription using the Reverse Transcription system (TaKaRa Biotechnology Co., Ltd., Dalian, China) using 1 μg RNA and stored at -20°C. Reverse transcription of microRNA was performed following the addition of a polyA tail.

To test the expression of miR-16 in tissues, SYBR® PrimeScript™ miRNA RT-PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was used, with U6 acting as internal reference. The RT-qPCR reaction system (30 μL) contained

5 μL cDNA, 10 μL Mix, 1 μL upstream primer (miR-16: 5'-TAGCAGCACGTAAATATTGG-3'; U6: 5'-CTCGCTTCGGCAGCACA-3'), 1 μL downstream universal primer (miR-16: 5'-CCAGTATTAACTGTGCTGCTGA-3'; U6: 5'-AACGCTTCACGAATTTGCGT-3') and 13 μL ddH_2O (Kapa Biosystems, Inc., Wilmington, MA, USA). Each sample was tested in triplicate. The PCR protocol was as follows: pre-denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 30 sec, and annealing at 60°C for 30 sec on the iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miR-16 against U6[19].

Cell-Counting Kit 8 (CCK-8) Assay

For the growth curve assay, A549 cells were seeded into 96-well plates at a density of 2,000 cells/well in triplicate. At 24 h, 48 h, and 72 h, cells were incubated with CCK-8 reagent (Cell Counting Kit-8; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min. Absorbance at 490 nm was read on a microplate reader (168-1000; Model 680, Bio-Rad Laboratories, Inc., Hercules, CA, USA), and proliferation curves were plotted using absorbance values at each time point.

Transwell Assay

Transwell chambers (8 μm diameter and 24 wells; Corning Inc., Corning, NY, USA) were used to evaluate the migration ability of A549 cells. Transfected cells were collected by trypsin digestion and resuspended to a density of 1×10^5 cells/mL in DMEM. Cell suspension (200 μL) was added to the upper chamber. In the lower chamber, 600 μL of DMEM supplemented with 10% FBS was added. Following incubation at 37°C for 24 h, cells in the upper chamber were removed by cotton swab. The chamber was subsequently fixed using 4% formaldehyde for 10 min at room temperature and subjected to Giemsa staining for 1 min. Following washing with phosphate-buffered saline (PBS) for three times, penetrating cells were counted under a light microscope (five fields; magnification, ×100) to evaluate migration ability.

Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) were used to determine the invasion ability of cells. Matrigel was diluted with serum-free DMEM at a ratio of 1:2. In the upper chamber, 50 μL of diluted Matrigel was added and kept at 37°C for 1 h. In the lower chamber, 600 μL of DMEM supplemented with 10% FBS was added. Following 24 h incubation, cells in the upper chamber were removed using a cotton swab. Subsequently, the chamber was fixed using 4% of formaldehyde for 10 min at room temperature and subjected to Giemsa staining for 1 min. After PBS washing for three times, penetrating cells were counted under a light microscope (five fields; magnification, ×100) to evaluate invasion ability.

Flow Cytometry

At 24 h after transfection, 1×10^6 cells in each group were washed twice with precooled PBS. Cell Cycle assay kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to evaluate the cell cycle according to the manufacturer's manual. Cells were incubated with 200 μL of liquid A for 10 min, and 150 μL of liquid B for another 10 min. Then, cells were incubated with 120 μL of liquid C in the dark for 10 min prior to flow cytometry (FACSVerse™; BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed using ModFit software (version 3.2; Verity Software House, Topsham, ME, USA).

Western Blotting

At 48 h after transfection, cells were digested and collected. Subsequently, precooled Radio-Immunoprecipitation assay lysis buffer (600 μL; 50 mM Tris-base, 1 mM ethylene diamine tetraacetic acid (EDTA), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, and 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) and phenylmethylsulfonyl fluoride were added to the samples. Following lysis for 5 min on ice, the mixture was centrifuged at 10,000 × g for 10 min at 4°C. The protein samples (50 μg) were then mixed with equal volume of 2x sodium dodecyl sulfate loading buffer prior to denaturation in a boiling water bath for 10 min. Subsequently, the protein samples (5 μL) were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. Resolved proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) on ice (300 mA, 2 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. The membranes were then incubated with rabbit anti-human matrix metalloproteinase (MMP)-19 polyclonal primary antibody (1:1,000 dilution; cat. no. ab53146) and rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (1:5,000

dilution; ab9485) at 4°C overnight. Following washing with Phosphate-Buffered Saline with Tween 20 for five times, with 5 min each time, membranes were incubated with goat anti-mouse (1:5,000 dilution; ab6789) and goat anti-rabbit (1:2,000 dilution; ab6721) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. All antibodies were purchased from Abcam (Cambridge, MA, USA). The membrane was developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for imaging. Image lab v3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of MMP-19 was expressed as MMP-19/GAPDH.

Dual Luciferase Reporter Assay

The potential target genes of miR-16 were predicted by searching the term "miR-16" on the TargetScan website (targetsacn.org). MiR-16 was identified to be capable of binding with the 3′-UTR of MMP-19 mRNA. The expression of MMP-19 was altered by transfection with the small interfering (si)RNA of MMP-19 (siR-MMP-19; HanBio Biotechnology Co., Ltd., Shanghai, China) or miR-16 mimics. According to bioinformatics results, wild-type (WT) and mutant-type (MUT) sequences of miR-16 in the 3'-UTR of MMP-19 gene were chemically synthesized *in vitro*, underwent addition of *Spe*I and *Hind*III restriction sites and were cloned into pMIR-REPORT luciferase reporter plasmids (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A549 cells were co-transfected with plasmids (0.5 μg) containing WT or MUT 3'-UTR DNA sequences and miR-16 mimics. Following the cultivation at 37°C for 24 h, cells were lysed using a dual-luciferase reporter assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Luciferase intensity was measured using a GloMax 20/20 luminometer (Promega Corporation, Madison, WI, USA). Renilla luciferase activity was utilized as internal reference for calculating the luciferase activity of each sample.

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 16.0 statistical software (IBM Corp, Armonk, NY, USA). All measurement data were expressed as the mean ± standard deviations. Intergroup comparison was performed using a paired *t*-test and $p<0.05$ was considered as statistically significant.

Results

Low Expression of MiR-16 in NSCLC is Closely Associated with Metastasis and Clinical Stage

To detect the expression of miR-16 in NSCLC tissues, RT-qPCR was performed. The data indicated that miR-16 levels in tumor tissues were significantly lower than in tumor-adjacent tissues ($p<0.05$; Figure 1A). In addition, miR-16 levels in NSCLC patients with higher clinical stage were significantly higher than those with lower clinical stage ($p<0.05$; Figure 1B). Similarly, the expression of miR-16 in NSCLC

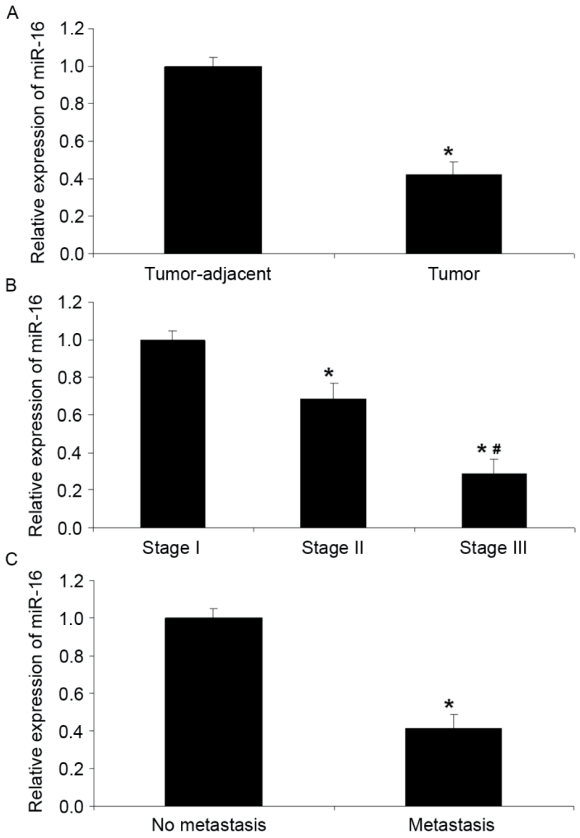

Figure 1. Expression of miR-16 in NSCLC and its clinical significance. ***A,*** Relative expression of miR-16 in tumor tissues and tumor-adjacent tissues. $^{*}p<0.05$ *vs.* tumor-adjacent tissues. ***B,*** Relative expression of miR-16 in patients with clinical stages I, II, and III NSCLC. $^{*}p<0.05$ *vs.* Stage I; $^{\#}p<0.05$ *vs.* Stage II. ***C,*** Relative expression of miR-16 in NSCLC patients with and without lymph node metastasis. $^{*}p<0.05$ *vs.* patients without lymph node metastasis. Data were expressed as the mean + standard deviation. NSCLC, non-small cell lung carcinoma; miR, microRNA.

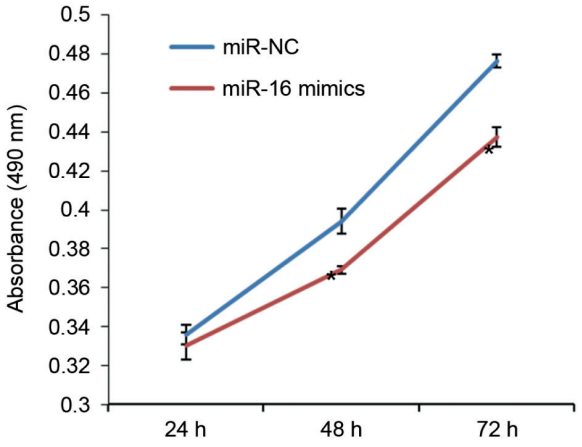

Figure 2. Effect of miR-16 on the proliferation of A549 cells *in vitro*. A549 cells were transfected with miR-16 mimics or miR-NC. A CCK-8 assay was used to measure the absorbance (490 nm) of the cells at 24, 48, and 72 h, and a cell proliferation curve was plotted. Data were presented as the mean ± standard deviation. $^{*}p<0.05$ *vs.* miR-NC. miR, microRNA; NC, negative control; CCK-8, cell counting kit 8.

patients with lymph node metastasis was significantly lower than those without lymph node metastasis ($p<0.05$; Figure 1C). These results suggested that the low expression of miR-16 in NSCLC is closely associated with a higher clinical stage and metastasis.

Overexpression of MiR-16 Inhibits the Proliferation of A549 Cells In Vitro

To determine the effect of miR-16 on the proliferation of A549 cells, CCK-8 assay was conducted. The data indicated that A549 cells transfected with miR-16 mimics exhibited significantly lower absorbance at 490 nm compared with those transfected with miR-NC at 48 and 72 h, respectively (both $p<0.05$; Figure 2). The result indicated that overexpression of miR-16 inhibited the proliferation of A549 cells *in vitro*.

MiR-16 Inhibits the Migration and Invasion of A549 Cells In Vitro

To examine the migration and invasion of A549 cells, transwell assay was performed. Migration assay indicated that the number of cells in miR-16 group migrating through the chamber membrane was significantly lower than that of the miR-NC group ($p<0.05$). Similarly, invasion assay demonstrated that the number of invaded cells in miR-16 group was significantly lower than that in the miR-NC group ($p<0.05$; Figure 3). The results suggested that miR-16 inhibited the migration and invasion of A549 cells *in vitro*.

Elevated miR-16 Expression Inhibits the Proliferation of A549 Cells by Regulating the G1/S Phase Transition

To study the cell cycle of A549 cells, flow cytometry was performed. The data demonstrated that the percentage of A549 cells overexpressing miR-16 in G1 phase was significantly higher than that of A549 cells transfected with miR-NC in G1 phase ($p<0.05$; Figure 4). In addition, the percentage of cells in G1 phase of the miR-NC or miR-16 groups was significantly higher than that of the cells in S phase ($p<0.05$; Figure 4). The result indicated that elevated miR-16 expression inhibited the proliferation of A549 cells by inhibiting the G1/S phase transition.

MiR-16 Exerts its Biological Function by Regulating the Expression of MMP-19

To assess the effect of miR-16 on the protein expression of MMP-19 in A549 cells, Western blotting was performed. Western blots indicated that the expression of MMP-19 in A549 cells overexpressing miR-16 was significantly lower than that of the miR-NC group ($p<0.05$; Figure 5). This suggested that miR-16 may exert its biological functions by regulating MMP-19 expression.

MiR-16 Binds to the 3′-UTR Region of MMP-19 mRNA

To identify the interaction between miR-16 and the 3′-UTR of MMP-19 mRNA, dual-luciferase reporter gene assay was performed. Luciferase activity in cells co-transfected with miR-16 mimics and pMIR-REPORT-WT luciferase reporter plasmids was significantly lower than that of the NC group ($p<0.05$). By contrast, luciferase activity in cells co-transfected with miR-16 mimics and pMIR-REPORT-mutant luciferase reporter plasmids did not differ significantly from that of the NC group (Figure 6). This indicated that miR-16 can bind to the 3′-UTR region of MMP-19 mRNA.

Discussion

MicroRNAs are capable of regulating post-transcriptional gene expressions. They are closely associated with the occurrence and development of almost all types of tumors[20]. Due to the identification of tumor-associated microRNAs, the functions and molecular mechanisms of microRNAs in

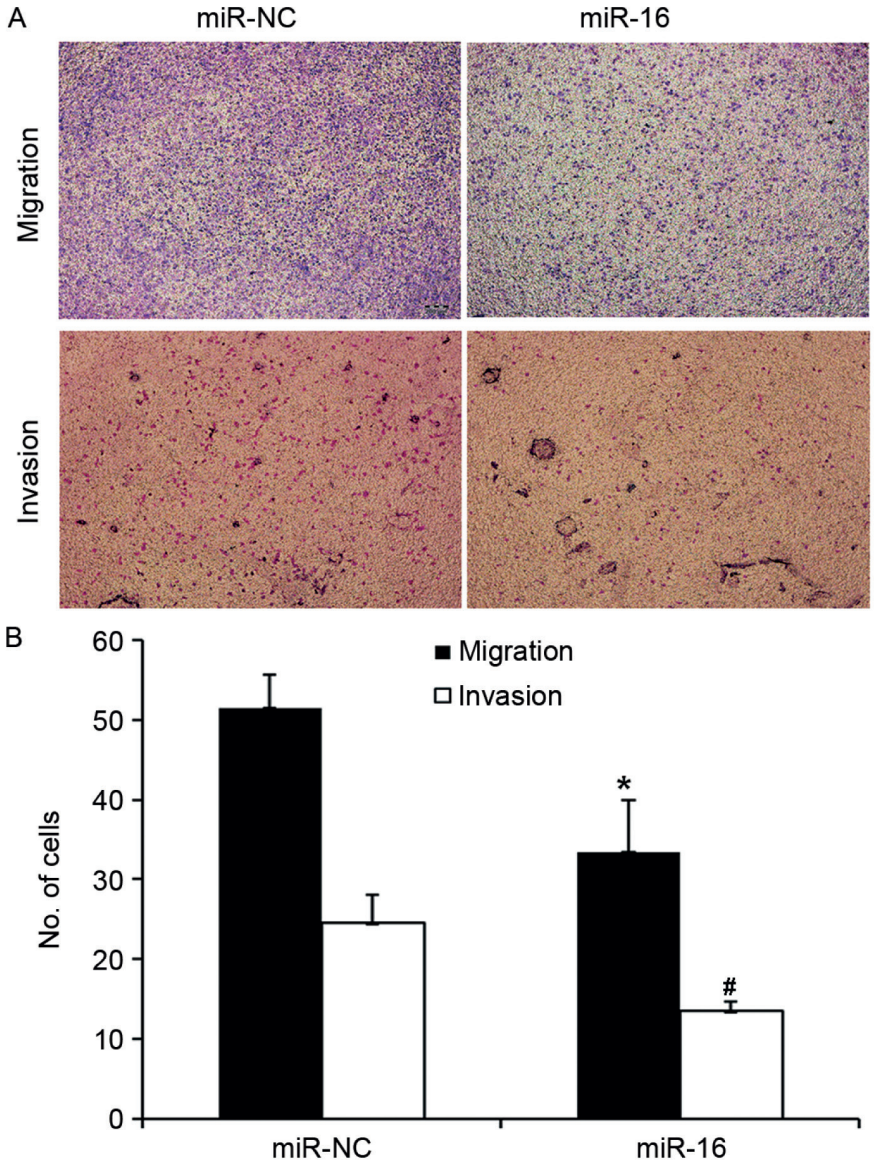

Figure 3. Effect of miR-16 on the migration and invasion of A549 cells *in vitro*. A549 cells were transfected with miR-16 mimics or miR-NC and the migration and invasion abilities of the cells were determined using a Transwell assay. Magnification, ×100. Data were presented as the mean ± standard deviation. $^{*}p<0.05$ *vs.* migration miR-NC group; $^{\#}p<0.05$ *vs.* invasion miR-NC group. miR, microRNA; NC, negative control.

tumors have been determined. These microRNAs may be useful in the early diagnosis and treatment of tumors[21]. MiR-16 is a tumor-associated molecule and exhibits abnormal expression in many types of tumor[22]. MiR-16 is closely associated with various biological processes including proliferation, drug resistance, invasion, and metastasis of tumor cells. He et al[23] have demonstrated that miR-16 regulates the phosphatidylinositol 3-kinase/AKT and MAPK signaling pathways by down-regulating fibroblast growth factor 2 expression and inhibits the proliferation, invasion, and metastasis of nasopharyngeal carcinoma cells. In addition, Han et al[24] determined that miR-16 increases the temozolomide-resistance of glioma cells by targeting Bcl-2. In the present investigation, the expression of miR-16 was down-regulated in NSCLC and that the decreased expression of miR-16 was associated with lymph node metastasis and TNM clinical staging. This suggested that miR-16 serves a tumor-suppressor role in NSCLC, similar to its role in melanoma, ovarian cancer, and colon cancer[25,26]. The *in*

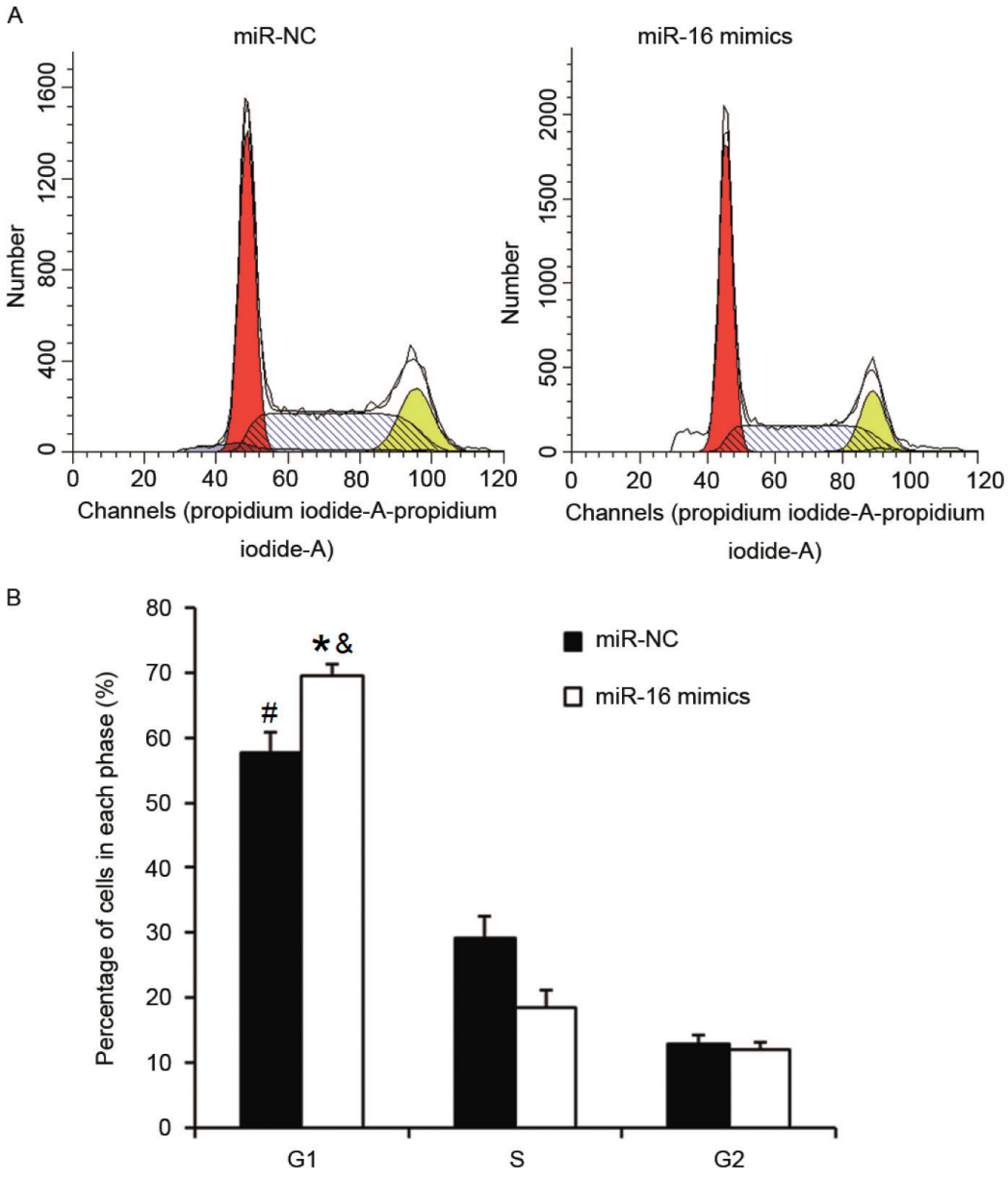

Figure 4. Effect of miR-16 on the A549 cell cycle. ***A,*** Cell cycle determination by flow cytometry. ***B,*** Percentages of cells in G1, S and G2 phases of the cell cycle. Data were presented as the mean + standard deviation. miR, microRNA; NC, negative control. $^{*}p<0.05$ compared with G1 phase of miR-NC group; $^{\#}p<0.05$ compared with S phase of miR-NC group; $^{\&}p<0.05$ compared with S phase of miR-16 mimics group.

vitro experiments performed in the current study indicate that overexpression of miR-16 inhibited the proliferation, G1/S phase transition, invasion, and metastasis of A549 cells and that the decreased expression of miR-16 promoted the development of NSCLC.

MicroRNAs exert their biological functions primarily by binding to the 3′-UTR of the mRNA of their target genes and inhibiting translation of the target gene[27]. Bioinformatics indicates that MMP-19 is a target gene of miR-16. MMP-19 is a member of the matrix metalloproteinase family, and it has been demonstrated that MMP-19 expression is up-regulated in a number of tumors, thus promoting the invasion and metastasis of tumor cells[28]. Lee et al[29] determined that MMP-19 enhances the expression of Slug and promotes the epithelial-mesenchymal transition in gallbladder

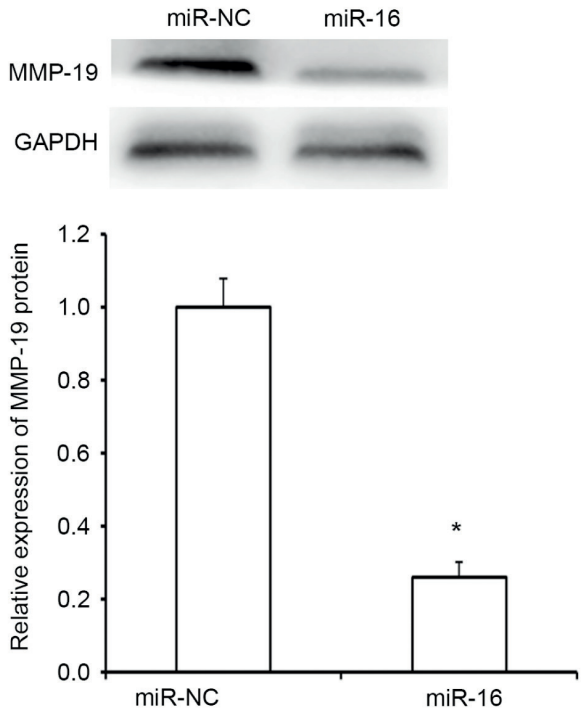

Figure 5. Effect of miR-16 on the expression of MMP-19 in A549 cells. Western blotting was used to measure protein expression. Data were presented as the mean + standard deviation. $^{*}p<0.05$ *vs.* miR-NC. MMP-19, matrix metalloproteinase 19; miR, microRNA; NC, negative control.

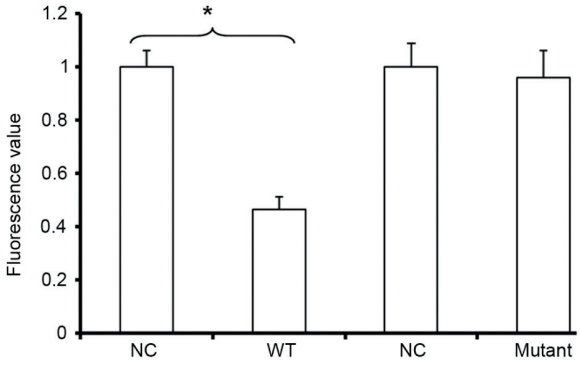

Figure 6. Identification of the interaction between miR-16 and MMP-19 using a dual luciferase reporter assay. A549 cells were co-transfected with plasmids (0.5 μg) with WT or MUT 3'-UTR DNA sequences and miR-16 mimics. Following the cultivation for 24 h, cells were lysed using a dual luciferase reporter assay kit, and the luciferase intensity was measured with the GloMax 20/20 luminometer. Using Renilla luciferase activity as an internal reference, the luciferase values of each group of cells were measured. Data were presented as the mean + standard deviation. $^{*}p<0.05$. miR, microRNA; NC, negative control; WT, wild-type; 3′UTR, 3′ untranslated region; MMP, matrix metalloproteinase.

carcinoma. Yu et al[30] reported that the overexpression of MMP-19 promotes the invasion and metastasis of NSCLC. Our results demonstrated that the overexpression of miR-16 down-regulated the MMP-19 expression in A549 cells. Based on the results of the aforementioned studies, it is hypothesized that the down-regulation of MMP-19 by miR-16 may be the mechanism that reduces the invasion and migration of A549 cells. Indeed, the dual-luciferase reporter gene assay demonstrated that miR-16 directly bound with the 3′-UTR of MMP-19 mRNA.

Conclusions

The present study demonstrated that down-regulation of miR-16 expression is associated with the occurrence and development of NSCLC. MiR-16 down-regulates the expression of MMP-19, inhibits the proliferation, invasion, and metastasis of NSCLC. Thus, miR-16 may be developed as a diagnostic marker and target of novel therapeutic strategies to treat NSCLC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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