MicroRNA-219 is downregulated in non-small cell lung cancer and inhibits cell growth and metastasis by targeting HMGA2

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Abstract. Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-associated mortality worldwide. Non-small cell lung cancer (NSCLC) is the predominant type of lung cancer, and accounts for ~85% of all lung cancer cases. An increasing number of studies suggest that microRNAs (miRs) may be involved in the regulation of NSCLC carcinogenesis and progression. However, the expression and function of miRNA-219 in NSCLC, and its underlying mechanisms of action, remain unknown. In the present study, miR-219 expression in NSCLC tissues and cell lines was determined using reverse transcription-quantitative polymerase chain reaction. Following transfection with miR-219 mimics, the effects of miR-219 overexpression on NSCLC cell proliferation, migration and invasion were examined. Furthermore, the miR-219 target in NSCLC was investigated. miR-219 was observed to be downregulated in NSCLC tissues and NSCLC cell lines. In addition, miR-219 was demonstrated to function as a tumor suppressor in NSCLC, through inhibiting cell proliferation, migration and invasion in vitro. Furthermore, high mobility group AT-hook 2 (HMGA2) was identified to be a direct target of miR-219 in NSCLC, and downregulation of HMGA2 suppressed NSCLC cell proliferation, migration and invasion in vitro. HMGA2 expression was upregulated in NSCLC tissues, and was inversely correlated with miR-219 expression. In conclusion, miR-219 functions as a tumor suppressor and may be important in inhibiting the growth and metastasis of NSCLC cells via directly targeting HMGA2. Therefore, miR-219 may present a potential novel therapeutic target for NSCLC.

Introduction

Lung cancer is the most commonly diagnosed cancer and is the leading cause of cancer-associated mortality worldwide (1). A number of risk factors for lung cancer have been identified, including environmental pollution, smoking and occupational carcinogens, including polycyclic aromatic hydrocarbons, asbestos, chromium, arsenic and nickel compounds (2-4). Non-small cell lung cancer (NSCLC) is the predominant type of lung cancer, which accounts for ~85% of all lung cancer cases (5). There are four histologic subtypes of NSCLC, including adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma and large cell carcinoma (6). At present, the standard treatments for NSCLC are surgery followed by adjuvant radiation therapy and chemotherapy (7). Despite extensive developments in prevention, diagnosis and treatment strategies, the prognosis of patients with NSCLC has not improved significantly over the last several decades, as the five-year survival rates are 11% (8). Therefore, investigating the molecular mechanisms underlying tumor development, growth and metastasis is essential for identifying novel therapeutic targets for NSCLC for the development of more effective treatment strategies.

MicroRNAs (miRNAs/miRs) are an abundant class of endogenous, non-coding, short RNA sequences, which are ~19-25 nucleotides in length (9). miRNAs negatively regulate the expression of target genes through base-pairing with the 3'-untranslated region (3'-UTR), which subsequently results in cleavage of the target mRNA sequence and/or inhibition of translation (10). In this way, miRNAs serve important roles in various biological processes, including tumor cell proliferation, cell cycle progression, apoptosis, differentiation, angiogenesis, migration, and tumor cell invasion and metastasis (11). Of particular note, a number of previous studies have demonstrated that dysregulation of miRNA expression is correlated with carcinogenesis and the progression of human cancers (12-14). A number of miRNAs function as tumor suppressors to suppress the carcinogenesis and progression of several human cancers via downregulation of oncogenes (15-17). For instance, miR-494 suppressed the proliferation and induced apoptosis of ovarian cancer cells, through negatively regulating the fibroblast growth factor receptor 2 oncogene (18). By contrast, several highly expressed miRNAs function as oncogenes during tumor development.
by repressing tumor suppressor genes (19-21). For instance, miR-130a enhances the proliferation and migration of gastric cancer cells by targeting transforming growth factor β receptor (TGFβR) 2 (22). Therefore, miRNAs may serve as therapeutic targets for the development of novel treatment strategies against NSCLC.

In the present study, the expression levels and functional role of miR-219 in NSCLC cells was investigated, and its underlying molecular mechanisms of action were explored. The results demonstrated that miR-219 was downregulated in NSCLC tissues and cell lines. In addition, the high mobility group AT-hook 2 (HMGA2) was confirmed to be a direct target gene of miR-219 in NSCLC cells. The results suggest that miR-219 functions as a tumor suppressor in NSCLC via targeting HMGA2.

Materials and methods

Tissue specimens. The methods employed to acquire all patient tissue samples were approved by Ethical Committee of Weifang People's Hospital (Weifang, China), and all patients provided written informed consent. A total of 32 paired NSCLC tissues and adjacent normal tissues were obtained from patients (male, 19; female, 13; age <55 years, 10; age ≥55 years, 22; mean age, 62 years; Tumor stage T1-T2, 14; T3-T4, 18; Tumor grade I-II, 15; III: 17) admitted to Weifang People's Hospital between 2012 and 2014. None of the patients recruited to the study were treated with chemotherapy or radiotherapy prior to surgery. Tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C until required.

Cell lines and culture conditions. The NSCLC cell lines, A549, SPC-A1, Calu-3, H460 and H1299, and the normal bronchial epithelial cell line, 16HBE, were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37°C in a humidified 5% CO₂ incubator.

Transfection of miRNA mimics, small interfering (si)RNAs and plasmids. miR-219 mimics, negative control and luciferase reporter plasmids were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). siRNAs targeting HMGA2 and negative control siRNAs were synthesized by Guangzhou Ribobio Co., Ltd. (Guangzhou, China): miR-219 mimic sequence, 5'-UGAUGUUGCAACCCAGAGUUCU-3'; NC sequence, 5'-UUCUCCGAACGUUGUGACACGTT-3'; HMGA2 siRNA sequence, 5'-CGGCGAAGAGCAGACCUATT-3'; NC siRNA sequence, 5'-UUCUCCGAACGUUGACACGTT-3'. Cells were seeded into six-well plates at a density of 8x10⁵ cells/well. Following overnight incubation, cells were transfected with miR-219 mimics (50 pmol/ml), NC (50 pmol/ml), HMGA2 siRNA (50 pmol/ml) or NC siRNA (50 pmol/ml) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following transfection for 6 h, the medium was replaced with fresh DMEM medium containing 10% FBS.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed to cDNA using the M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. SYBR® Green PCR Master Mix (Toyobo, Osaka, Japan) was used to measure the expression of miR-219 and HMGA2 mRNA, with U6 and GADPH as the internal controls, respectively. The following thermocycling conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. qPCR was performed using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were designed as follows: miR-219, 5'-GGGGCGGCGUCUC-3' (forward) and 5'-GCCCAAAACCU CGAGCGG-3' (reverse); U6, 5'-CGCAAGGATGACCG CAAATTC-3' (forward) and 5'-GTGCGAGGCGCCGAGT-3' (reverse); HMGA2, 5'-GCCGACATTTTCAAGGGCAC-3' (forward) and 5'-GCTGACACCATCAACCC-3' (reverse); GAPDH, 5'-ACCACAGTCTGAGCCATCC-3' (forward) and 5'-TCCACACCCCTTGTGGTCGTA-3' (reverse). The relative fold-change in target gene expression was determined using the 2ΔΔCq method (23).

MTT assay. A549 and H460 cells were seeded in 96-well plates at a density of 3,000 cells/well. Following overnight incubation, cells were transfected as aforementioned. After transfection, an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was performed to determine cell viability every 24 h over the course of four consecutive days. Briefly, 20 μl MTT solution (5 mg/ml) was added to each well, and cells were incubated at 37°C for 4 h in a humidified atmosphere with 5% CO₂. The culture medium containing MTT solution was removed carefully, and 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well to dissolve the formazan crystals. The optical density at 490 nm was read using a VersaMax Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA).

Cell migration and invasion assay. At 48 h following transfection with miR-219 mimics, HMGA2 siRNA or their respective negative controls, the A549 and H460 cells were harvested. In order to perform cell migration analysis, 4x10⁴ cells suspended in 100 μl FBS-free medium were added to the upper chamber of a transwell plate (8-mm pore size; BD Biosciences, San Jose, CA, USA). For the cell invasion assay, 4x10⁴ cells suspended in 100 μl FBS-free medium were added into the upper chamber of transwell chambers coated with Matrigel (BD Biosciences). A total of 600 μl DMEM medium containing 10% FBS was added into the lower chambers. Cells were incubated at 37°C for 48 h in a humidified atmosphere at 5% CO₂. Any cells that remained on the upper chamber were removed carefully with a cotton swab. Migrated or invaded cells that had traversed the membrane were fixed in 95% methanol for 10 min and stained with 0.5% crystal violet for 10 min at room temperature. The cells were subsequently photographed and counted in five randomly selected visual fields under an inverted microscope (x200 magnification; Olympus Corporation, Tokyo, Japan).
Bioinformatics analysis and luciferase reporter assay. The following three miRNA target prediction programs were used to identify the predicted target genes of miR-219: microRNA.org (August 2010 Release; http://www.microrna.org/) (24), miRDB version 4.0 (http://mirdb.org/) (25,26) and TargetScan version 6.2 (http://www.targetscan.org/) (27).

A549 and H460 cells were seeded in 24-well plates at a density of 1.5x10³ cells per well, and cultured at 37°C in a humidified atmosphere and 5% CO₂. When the cell density reached 60-70%, cells were transfected with wild-type pMIR-HMGA2-3′UTR Wt or mutant pMIR-HMGA2-3′UTR Mut luciferase reporter plasmids, together with miR-219 mimics or negative control miRNAs, using Lipofectamine 2000. Transfected cells were incubated for 48 h, and luciferase activity was detected using the Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer’s instructions. All experiments were performed in triplicate.

Western blot analysis. A549 and H460 cells were transfected as aforementioned with miR-219 mimic, HMGA2 siRNA or the respective negative controls. The cells were harvested at 72 h post-transfection using radioimmunoprecipitation lysis buffer supplemented with a complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using a bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein (30 µg) were resolved on a 10% SDS-PAGE gel, and then transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked in 5% nonfat milk for 1 h at room temperature, before they were probed with mouse monoclonal anti-human HMGA2 (dilution, 1:1,000; cat. no. ab84616; Abcam, Cambridge, UK) and mouse anti-human monoclonal GADPH (dilution, 1:1,000; cat. no. ab8245; Abcam) primary antibodies at 4°C overnight. Following washing with Tris-buffered saline and 0.1% Tween 20, the membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (dilution, 1:10,000; cat. no. ab6789; Abcam) for 2 h at room temperature. The protein bands were detected using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.), GADPH was used as an internal control for determining HMGA2 protein expression.

Statistical analysis. Results are presented as the mean ± standard deviation. Data were analyzed using a Student’s t-test or one-way analysis of variance followed by the Newman-Keuls method. Spearman’s correlation analysis was used to explore the association between miR-219 and HMGA2 mRNA expression level. Statistical analysis was performed using SPSS software version 19.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-219 is downregulated in NSCLC tissues and cell lines. In order to evaluate the biological role of miR-219 in NSCLC, its expression in NSCLC tissues and normal adjacent tissues was first assessed. Results from the RT-qPCR analysis demonstrated that miR-219 was significantly downregulated in NSCLC tissues when compared with normal adjacent tissues (P<0.05; Fig. 1A). Similarly, miR-219 expression levels in five NSCLC cell lines were significantly reduced when compared with the normal bronchial epithelial cell line, 16HBE (P<0.05; Fig. 1B). These results suggest that miR-219 may be frequently downregulated in NSCLC tissues and cell lines, and its downregulation may contribute to NSCLC occurrence and development.

miR-219 inhibits NSCLC cell proliferation, migration and invasion in vitro. To explore the biological function of miR-219 in NSCLC, miR-219 mimics were transfected into A549 and H460 cells to increase its expression. As shown in Fig. 2A, the expression levels of miR-219 were significantly increased in A549 and H460 cells transfected with miR-219 mimics when compared with negative controls (P<0.05). MTT and cell migration and invasion assays were then performed to investigate the effect of miR-219 overexpression on NSCLC cell viability, migration and invasion, respectively. The results of the MTT assay revealed that upregulation of miR-219 induced a significant decrease in A549 and H460 cell viability at 72 and 96 h following transfection when compared with negative controls (P<0.05; Fig. 2B). In addition, overexpression of miR-219 significantly suppressed the migratory and invasive capacities of A549 and H460 cells (P<0.05; Fig. 2C). These results demonstrated that miR-219 might serve a significant role in regulating the growth and metastatic potential of NSCLC cells in vitro.
HMGA2 is a direct target of miR-219. In order to investigate the molecular mechanisms underlying the anti-tumor effects of miR-219 in NSCLC, bioinformatics analysis was performed using three independent miRNA target prediction programs. As shown in Fig. 3A, a conserved sequence in the 3'-UTR of HMGA2 mRNA was identified to be complementary to the seed region of miR-219. To determine whether HMGA2 may be a direct target of miR-219, a luciferase reporter assay was performed. As shown in Fig. 3B, miR-219 significantly suppressed the luciferase activity in A549 and H460 cells transfected with pMIR-HMGA2-3'UTR Wt, whereas no significant alterations in luciferase activity were observed in pMIR-HMGA2-3'UTR Mut-transfected cells when compared with negative controls. The effect of miR-219 overexpression on the protein and mRNA levels of HMGA2 in NSCLC cells was then investigated. Western blotting analysis demonstrated that miR-219 overexpression significantly reduced HMGA2 protein expression levels in A549 and H460 cells when compared with negative controls (P<0.05; Fig. 3C). By contrast, overexpression of miR-219 demonstrated no significant effect on the mRNA expression levels of HMGA2 when compared with negative controls (Fig. 3D). These results suggest that HMGA2 may be a novel direct target of miR-219.

HMGA2 knockdown inhibits the proliferation, migration and invasion of NSCLC cells. To determine whether HMGA2 knockdown, similar to miR-219 overexpression, was associated with inhibition of NSCLC cell growth and metastatic behaviors, the effects of HMGA2 silencing on NSCLC cell proliferation, migration and invasion were examined. A549...
and H460 cells were first transfected with HMGA2 or negative control siRNAs. At 72 h post-transfection, western blotting analysis revealed that HMGA2 expression was significantly downregulated in HMGA2 siRNA-transfected A549 and H460 cells when compared with negative controls (P<0.05; Fig. 4A). Downregulation of HMGA2 expression significantly inhibited the viability of A549 and H460 cells at 72 and 96 h following transfection, and significantly reduced the migration and invasion capabilities of these cells when compared with negative controls (P<0.05; Fig. 4B and C). These results suggest that suppression of HMGA2 expression by miR-219 may serve an important role in reducing NSCLC cell growth and metastasis.

HMGA2 is inversely correlated with miR-219 expression in NSCLC. The expression levels of HMGA2 mRNA in NSCLC tissues and adjacent normal tissues were assessed by RT-qPCR analysis. As shown in Fig. 5A, HMGA2 mRNA was significantly upregulated in NSCLC tissues when compared with adjacent normal tissues (P<0.05). Spearman's correlation analysis revealed a significant inverse correlation between the expression of HMGA2 mRNA and miR-219 in NSCLC tissues (r=-0.7512; P<0.001; Fig. 5B). These results provide additional evidence to suggest that HMGA2 may be a direct target of miR-219.

Discussion

miR-219 has been reported to be significantly deregulated in a number of human cancers. In hepatocellular carcinoma, miR-219 was downregulated in tumor tissues, and reduced miR-219 expression was correlated with tumor size, histological differentiation, and overall survival time (28). Huang et al (29) reported that the expression level of miR-219 was reduced in papillary thyroid carcinoma tissues, and low miR-219 expression was associated with sex, tumor size, and lymph node metastasis in patients with papillary thyroid carcinoma. Xiong et al (30) observed that miR-219 was lower in colorectal cancer tissues when compared with corresponding matched normal tissues. In addition, downregulation of miR-219 has been reported in medulloblastoma (31), tongue squamous cells carcinoma (32) and colon cancer (33). However, to the best of the author's knowledge, no studies regarding the expression of miR-219 in NSCLC have been published to date. The results of the present study demonstrated that miR-219 is downregulated in NSCLC tissues and cell lines, which suggests that miR-219 may serve important roles in NSCLC.

Recently, a significant number of studies have focused on the role of miR-219 in tumorigenesis and the progression of multiple cancers. Huang et al (28) reported that miR-219 inhibited the proliferation of hepatocellular carcinoma cells in vitro, and lead to cell cycle arrest at the G1 to S transition via direct targeting of glypican-3. In addition, miR-219 was demonstrated to suppress the proliferation, anchorage-independent growth and migration of glioblastoma cells through negatively regulating the epidermal growth factor receptor (34). Furthermore, it was demonstrated that upregulation of miR-219 inhibited the proliferation and invasive capabilities of glioblastoma cells, enhanced apoptosis in vitro, and inhibited xenograft formation in vivo by targeting roundabout guidance receptor 1 (35). Shi et al (31) demonstrated that upregulation of miR-219 inhibited the proliferation, migration and invasion of
Figure 4. HMGA2 knockdown inhibits the viability, migration and invasion of NSCLC cells in vitro. (A) HMGA2 protein expression in A549 and H460 cells transfected with HMGA2 or NC siRNA, as determined by western blot analysis. (B) The viability of A549 and H460 NSCLC cells transfected with HMGA2 or NC siRNA at 24, 48, 72 and 96 h using an MTT assay. (C) The migration and invasion capabilities of A549 and H460 cells transfected with HMGA2 or NC siRNA using transwell chambers pre-coated without or with Matrigel, respectively (magnification, x200). *P<0.05 vs. NC siRNA. HMGA2, high mobility group AT-hook 2; NSCLC, non-small cell lung cancer; siRNA, NC, negative control; small-interfering RNA; OD, optical density.
miR-219 is a microRNA (miRNA) that can inhibit the expression of HMGA2, a high mobility group AT-hook 2 protein, in non-small cell lung cancer (NSCLC) tissues. HMGA2 is a transcription factor that can regulate cell proliferation, migration, and invasion. The expression of HMGA2 is inversely correlated with miR-219 expression in NSCLC tissues. This suggests that miR-219 may function as a tumor suppressor in NSCLC, partly by targeting HMGA2. The development of miR-219/HMGA2-based targeted therapies may present a potentially effective therapeutic strategy in NSCLC.

In conclusion, the results of the present study provide evidence to suggest that miR-219 may function as a tumor suppressor in NSCLC, partly by targeting HMGA2. The development of miR-219/HMGA2-based targeted therapies may therefore be a potential strategy for the treatment of NSCLC patients.

**References**


