MicroRNA-4500 suppresses tumor progression in non-small cell lung cancer by regulating STAT3

ZHI-YING LI1*, ZI-ZHOU ZHANG2*, HUI BI1, QIU-DI ZHANG1, SU-JUAN ZHANG1, LIN ZHOU1, XIAO-QIN ZHU1 and JUN ZHOU1

1Department of Respiratory Medicine, The First People’s Hospital of Changzhou, Changzhou, Jiangsu 213000; 2Department of Respiratory Medicine, The Seventh People’s Hospital of Changzhou, Changzhou, Jiangsu 213011, P.R. China

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Abstract. Research has revealed that microRNA (miR)-4500 is downregulated in non-small cell lung cancer (NSCLC), and miR-4500 suppresses tumor growth by targeting lin-28 homolog B and NRAS proto-oncogene, GTPase. In the present study, it was reported that signal transducer and activator of transcription 3 (STAT3) may function as a novel target gene for miR-4500 in NSCLC. The experiments conducted in the present study confirmed that the miR-4500 expression was decreased in NSCLC tissues and cells compared with adjacent normal tissues and a normal lung cell line. miR-4500 suppressed the cell proliferation, migration, invasion and promoted apoptosis of the human NSCLC cell lines A549 and H1975. Expression of STAT3 was negatively correlated with miR-4500 expression in vivo. A luciferase reporter assay suggested that miR-4500 directly targeted the 3' untranslated region of STAT3. The tumor inhibition effect of small interfering RNA STAT3 in A549 and H1975 lines may be partially impaired by a miR-4500 inhibitor. The results of the present study suggests that miR-4500 may be a tumor suppressor and a potential therapeutic target in NSCLC.

Introduction

Lung cancer is the main reason for cancer-associated mortality, causing >1.37 million cases of mortality globally annually (1,2). In China, the number of lung cancer cases has reached 800,000, and 700,000 cases of mortality have been caused by lung cancer in 2017. The 5-year survival rate of lung cancer is as low as ~18%, due to the fact that the majority of patients are in the middle or late stage when they are admitted for hospitalization and thus have lost the opportunity for surgical treatment (3). Non-small cell lung cancer (NSCLC) constitutes 85% of all lung cancer cases. The initiation and development of NSCLC is attributed to the aberrant expression of proto-oncogenes and tumor-suppressive genes, which result in tumor cell growth, metastasis and eventually tumor progression (1).

MicroRNAs (miRNAs/miRs) are short endogenous non-protein-coding RNAs that are ~22 nucleotides in length. miRNAs serve notable functions in regulating human gene expression by base pairing to the 3' untranslated region (3'UTR) of the target mRNA (4). Various miRNAs are involved in a diverse range of cellular processes, including cell proliferation, apoptosis, development and differentiation (5). Increasing evidence demonstrates that the dysregulation of miRNA expression is associated with the development and progression of various cancer types including NSCLC (6-10).

miR-4500, which is 16 nucleotides in length and is located in chromosome 13, was discovered by high-throughput sequencing technology (11). One previous study demonstrated that miR-4500 was downregulated in NSCLC lung tissues compared with their non-tumor counterparts, and that the low expression of miR-4500 promoted tumor growth by targeting lin-28 homolog B (LIN28B) and NRAS proto-oncogene, GTPase (NRAS) (12). Using a bioinformatic algorithm, signal transducer and activator of transcription proteins 3 (STAT3) was predicted to be a novel potential target of miR-4500 (13).

STAT3 has been demonstrated to be crucial in tumor development and cancer-associated inflammation (14). STAT3 may prevent apoptosis and promotes cell proliferation and angiogenesis (15). A number of miRNAs are known to interact with the 3'-UTR of STAT3 mRNA and thereby negatively regulate STAT3 (16-19). For example, miR-17, miR-20a and miR-106b control lung epithelial branching morphogenesis through the downregulation of STAT3 and mitogen-activated protein kinase 14 protein levels and followed by the regulation of E-Cadherin distribution (20). miR-9600 suppresses tumor progression and promotes paclitaxel sensitivity in NSCLC through altering STAT3 expression in NSCLC cell lines (18).
The present study initially investigated the expression of miR-4500 and STAT3 in NSCLC tissues and the human NSCLC cell lines A549 and H1975. Then, the function of miR-4500 in cell proliferation, migration, invasion and apoptosis in vitro was examined. Furthermore, the molecular mechanism of miR-4500 in tumor progression was studied. The results of the present study suggested that miR-4500 may serve a regulatory role in NSCLC progression, and may be a novel strategy and prognostic marker for the diagnosis and prognosis of NSCLC.

Materials and methods

Human tissue samples. The present study was ethically approved by the Ethics Committee of the First People's Hospital of Changzhou (Changzhou, China). Clinical samples (NSCLC tissues and adjacent normal tissues) were collected from 40 patients with NSCLC (average age, 57; female to male ratio, 17:23; 11 patients aged <60 years old and 29 patients aged ≥60 years old) who received surgery at The First People's Hospital of Changzhou (Changzhou, China) between July 2015 and November 2017 subsequent to obtaining written informed consent. All patient diagnoses of NSCLC were confirmed based on a pathological assay, and none of the patients received any prior cancer treatment.

Cell culture and transfection. Human NSCLC cell lines A549 and H1975 and a human normal lung cell line (16HBe), (FBS; Biowest, Riverside, Mo, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator at 37°C in 5% CO2. miR-4500 mimics, miRNA mimics-negative control (NC), miR-4500 inhibitor and miRNA inhibitor NC were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Small interfering RNA (siRNA) against human STAT3 miRNA and the control siRNA were synthesized by Guangzhou riboBio Co., ltd. (Guangzhou, China). Transfection was performed using Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, A549 and H1975 cells were seeded in 6-well plates at a density of 5.0x10^3 cells/well transfected with miRNA (100 nM) or siRNA (50 nM) when the cells reached 60‑70% confluence. Subsequently, cells were cultured with fresh medium containing 10% FBS for 48 h prior to further experiments.

The target sequences of the siRNA used are as follows: siRNA-STAT3 sense, 5'-CCAGTCACTGGACAGGCAGAA G-3' and antisense, 5'-GCACGTAATCCATCGCTGACA-3'.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed using a Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA) at 37°C for 30 min. miRNA was extracted using the miRcute miRNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China). cDNA was synthesized using the One Step PrimeScript miRNA cDNA Synthesis kit according to the manufacturer's protocol (Takara Bio, Inc., Otsu, Japan). The expression levels of miR-4500 and STAT3 miRNA were determined using RT-qPCR using the SYBR ExScript RT-qPCR kit according to the manufacturer's protocol (Takara Biotech Co., Ltd.) in the ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with U6 and GAPDH functioning as endogenous controls, respectively. The primers used for qPCR were as follows: STAT3 forward, 5'-ATACACGCTCTTCTACAGTGC-3' and reverse, 5'-CATCCTGGAGATTCTTACCAC-3'; GAPDH forward, 5'-CCAATCTGCCAACCCTTGC-3' and reverse 5'-ACCCCTTTGCTGTAGCA-3'; and U6 forward, 5'-CTCGTCTCGGACGACA-3' and reverse, 5'-AAGCCT TCACGAATTTGCGT-3'. The reaction system consisting of a total of 20 µl of volume was as follows: 1 µl cDNA, 10 µl SYBR Premix EX Taq, 1 µl each of the primers (10 µM) and 7 µl ddH2O. The thermocycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Melting curve analysis was performed at the end of each PCR cycle to confirm that only one product was amplified and detected. All fold changes were calculated using the comparative Cq (2 ΔΔcq) method using U6 or GAPDH for normalization (21).

Western blotting. Total protein was extracted from transfected cells using radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Protein concentration was measured with a Bicinchoninic acid Protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China). Protein concentration was measured with a Bicinchoninic acid Protein assay kit (Takara Bio, Inc., Otsu, Japan) at 37°C for 30 min. The expression levels of miR-4500 and STAT3 were examined. Furthermore, the molecular mechanism of miR-4500 in tumor progression was studied. The results of the present study suggested that miR-4500 may serve a regulatory role in NSCLC progression, and may be a novel strategy and prognostic marker for the diagnosis and prognosis of NSCLC.

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chemiluminescent kit (Beyotime Institute of Biotechnology, Beijing, China). The immunoreactive bands were scanned using a densitometer, and the gray value of the bands were calculated automatically using ImageJ software version 1.45 (National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay. Cell proliferation of A549 and H1975 was detected using the Cell Counting Kit-8 (CCK-8; Wuhan Boster Biological Technology, Ltd., Wuhan, China) according to the manufacturer's protocol. A colony formation assay and EdU assay were performed to confirm the CCK-8 assay results. For the EdU assay, cells were serum-starved for 24 h, prior to treatment. EdU was added to the culture 2 h prior to cell collection and detected using the Click-it assay kit (Thermo Fisher Scientific, Inc.). For the colony formation assay, cells were seeded in a 6-well plate at a density of 1,000 cells/well 24 h after transfection. Cells were incubated for 2 weeks and the clones were fixed at room temperature for 20 min using methanol and stained with 0.1% crystal violet for 4 h at room temperature, and counted under a light microscope.

Cell apoptosis assay. The apoptosis of A549 and H1975 cells was detected using flow cytometry analysis. A fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) Apoptosis Detection kit (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's protocol. All cells were collected 48 h post-transfection and stained using Annexin V-FITC (5 ml) and PI (5 ml) according to the manufacturer's protocol. Flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) was used for cell apoptosis determination and analyzed using FlowJo 10.06 software (FlowJo LLC, Ashland, OR, USA). All analyses were performed in triplicate.

Wound healing assay. A549 and H1975 cells were seeded in 6-well plates and then incubated with 10 µg/ml mitomycin C...
for 2 h. Wounds were created using a 200 µl sterile pipette tips when the density reached ~80%. Photographs were obtained at 24 h following wound generation under a light microscope (magnification, x200).

Transwell invasion assay. A549 and H1975 cells were plated in the upper Transwell chamber (Corning Incorporated, Corning, NY, USA) that was pre-coated with Matrigel (Growth factor reduced; BD Biosciences), at a density of 4x10⁴ cells/well in 100 µl DMEM (with 0.5% FBS). The bottom chambers of the Transwell chamber were filled with DMEM with 20% FBS. After 24 h at 37°C, fixed cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, cells were stained with 0.1% crystal violet for 30 min at room temperature. The migration and invasion cells were counted and imaged under a light microscope (magnification, x200).

Luciferase reporter assay. The primers used for pGL3-STAT3-3’-UTR construction were as follows: Sense, 5’-GGTaccGTGcTGcTcGTTTGCAGA-3’ and antisense, 5’-AAGCTTTCTAGCGATATTTCTTTGCAGA-3’. PCR products were cloned into the pGL3-basic vector using KpnI/HindIII sites. The mutant version of this construct (pGL3-STAT3-mut-3’UTR) carrying 4 base pair substitutions in the miR-4500 target sites was obtained by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer’s protocol. The A549 and H1975 cells were seeded at a concentration of 5.0x10³ cells/well onto 24-well plates and co-transfected with either miR-4500 mimics or miR-NC and pGL3-STAT3-3’UTR or pGL3-STAT3-mut-3’UTR using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 36 h at 37°C. After 36 h, luciferase activity was measured using the Dual-Glo Luciferase Assay System.
Renilla luciferase was used to normalize the luciferase activity.

**Statistical analysis.** All results are reported as the mean ± standard deviation and at least three independent replicates were performed. GraphPad software 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform the statistical analysis. Pearson's correlation analysis was used to assess the correlation between STaT3 mRNA expression and miR-4500 levels. Differences between two groups were calculated using a Student's t-test. Differences between multiple groups were analyzed using a one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**STAT3 is a putative target of miR-4500 in NSCLC.** Bioinformatics algorithms [TargetScan software (http://www.targetscan.org/)](22) implied that there was putative miR-4500 target site in the 3′-UTR of STAT3 (Fig. 1). Considering that STAT3 may be one of the key oncogenic drivers in NSCLC, the present study further investigated the association between miR-4500 and STAT3.

**miR-4500 expression is negatively correlated with STAT3 in NSCLC tissues and cell lines.** miR-4500 and STAT3 expression level were studied in 40 human NSCLC lung tissues. RT-qPCR results indicated that the expression levels of miR-4500 in NSCLC lung tissues were significantly lower compared with normal tissues (P<0.01; Fig. 2A). Conversely, STAT3 was significantly upregulated in NSCLC lung tissues on the mRNA and protein levels compared with normal tissues (P<0.05; Fig. 2B and C). Additionally, Pearson's correlation analysis was used to assess the correlation between STAT3 mRNA expression and miR-4500 levels in NSCLC lung tissues, and the results indicated that the expression levels of STAT3 mRNA and miR-4500 were significantly negatively correlated (r²=-0.5124, P<0.0001; Fig. 2D). Furthermore, NSCLC cell lines (A549 and H1975) also exhibited significantly lower
miR-4500 expression levels and higher STAT3 expression levels compared with a normal lung cell line, 16HBE (P<0.05; Fig. 2E and F).

miR-4500 directly targets STAT3 in NSCLC. To validate the interaction between STAT3 and miR-4500, luciferase reporter assays were performed. The results indicated that the overexpression of miR-4500 significantly decreased the luciferase activity of wild type STAT3-3’-uTr compared with mutated STAT3-3’-UTR (P<0.01), indicating that miR-4500 targeted the 3’-UTR of STAT3 directly in NSCLC (Fig. 3A).

miR-4500 suppresses NSCLC cell proliferation and invasion and induces apoptosis in vitro. In order to investigate the function of miR-4500 in cell proliferation and apoptosis, miR-4500 was overexpressed in A549 and H1975 cells by transfecting the cells with miR-4500 mimics. RT-qPCR results (Fig. 3B) indicated that a 2-4 fold significant increase in miR-4500 mRNA levels were expressed subsequent to transfection with mimics compared with the negative control (P<0.05). STAT3 expression was significantly decreased in A549 and H1975 cells following transfection with miR-4500 mimics (P<0.05; Fig. 3C). A CCK-8 assay indicated that miR-4500 mimics inhibited cell proliferation significantly when compared with the negative control at the 48 and 72 h mark (P<0.05; Fig. 4A). The same conclusion was derived from the colony formation assay (P<0.05; Fig. 4B) and the EdU assay (P<0.05; Fig. 4C). Furthermore, miR-4500 mimics significantly promoted cell apoptosis in A549 and H1975 cells compared with the negative control (P<0.05), with the anti-apoptotic protein Bcl2 significantly downregulated and pro-apoptosis proteins (Bax, cleaved-caspase-3 and cleaved-caspase-9) significantly upregulated compared with the negative control (P<0.05; Fig. 5).

A previous study has demonstrated the function of miR-4500 in NSCLC cell proliferation and apoptosis, with no data on cell migration and invasion (12). Therefore, a wound healing assay and a Transwell assay were performed in order to study the function of miR-4500 in NSCLC cell migration.
and invasion. The results revealed a significant positive effect of miR-4500 in migration and invasion inhibition compared with the NC group (P<0.05; Fig. 6A and B). MMPs participate in cancer cell invasion by degrading the extracellular matrix (23,24). Western blotting revealed that the protein expression levels of MMP-2 and MMP-9 were significantly inhibited in A549 and H1975 cells subsequent to transfection with miR-4500 mimics compared with the NC group (P<0.05; Fig. 6C).

miR-4500 suppresses NSCLC cell proliferation, migration and invasion and induces apoptosis partially by downregulating STAT3 in vitro. To identify the function of STAT3 in miR-4500-suppressed NSCLC cell progression, STAT3 expression was successfully significantly knocked down in A549 and H1975 cells by transfection with STAT3-targeted siRNA (P<0.05; Fig. 7A). It was revealed that STAT3 downregulation significantly suppressed cell proliferation (P<0.05), migration (P<0.05) and invasion (P<0.05) and significantly induced apoptosis (P<0.05) compared with the control. However, miR-4500 inhibitors partially restored the effects of si-STAT3 on cell growth and migration in A549 and H1975 cells (P<0.05; Figs. 7B-D and 8). This data implies that miR-4500 suppresses NSCLC cell proliferation, migration and invasion and induces apoptosis partially by downregulating STAT3 in vitro.

Discussion

miRNAs control various processes, including cellular growth, proliferation, differentiation, regulation of the cell cycle, aging, apoptosis, metabolism and neuronal patterning. An increasing number of miRNAs have been demonstrated to be associated with the development and progression of different types of cancer (25-29). All this indicates the potential function of miRNAs as novel diagnostic or prognostic biomarkers.

The let-7 family is one of the earliest identified miRNA families, originally determined to serve a function in the timing of larval development in Caenorhabditis elegans (30). The members of let-7 are highly expressed in normal lung tissues and have been identified to negatively control multiple different onco-genes (31). miRNA-4500 is one of the let-7 family members, and has been reported to be downregulated in NSCLC, consequently promoting tumor growth by targeting LIN28B and NRAS (32).
The present study confirmed the lower expression of miR-4500 in NSCLC tissues and cells compared with normal control tissues and cells. Cell lines A549 and H1975 were selected to study the role of miR-4500 in tumorigenesis. Results indicate that miR-4500 suppressed cell proliferation, migration, invasion and promoted apoptosis in vitro.

STAT3 is a well-known oncogenic gene in NSCLC (33-35). The constitutive activation of STAT3 is a common feature in...
nSclc, and has also been proposed to serve a notable function in tumor resistance to conventional and targeted small-molecule therapies (36-38). STAT3 suppresses cancer cell apoptosis via the inactivation of either extrinsic or intrinsic apoptotic pathways (39,40). Meanwhile, STAT3 promotes nSclc cell proliferation and angiogenesis (12,13).

Bioinformatic algorithms (TargetScan) implied that there was a putative miR-4500 target site in the 3'-UTR of STAT3. The present study revealed that the expression of STAT3 is negatively correlated with miR-4500 expression in the tissues of 40 patients with NSCLC. Furthermore, targeting miR-4500 and STAT3 was confirmed using a luciferase reporter assay. Additionally, the tumor inhibition effect of si-STAT3 in A549 and H1975 lines may be partially restored using a miR-4500 inhibitor, which suggests that miR-4500 may suppress tumor progression in NSCLC by regulating STAT3. In order to confirm this, further studies will also be performed in the future in cells overexpressing STAT3 and miR-4500.

Altogether, miR-4500, that was demonstrated to be significantly downregulated in NSCLC tissues and cell lines, may suppress NSCLC cell proliferation, migration and invasion, and promote apoptosis by regulating STAT3 expression, indicating that miR-4500 may be a tumor suppressor and a potential therapeutic target in NSCLC.

Figure 8. miR-4500 suppresses non-small cell lung cancer cell migration and invasion by downregulating STAT3 in vitro. (A) A Wound healing assay and (B) a Transwell invasion assay of A549 and H1975 cells subsequent to transfection with si-STAT3 or NC or a combination of si-STAT3 and miR-4500 inhibitors. (C) Western blotting determined the protein expression levels of MMP-2 and MMP-9 in A549 and H1975 cells transfected with with si-STAT3 or NC or a combination of si-STAT3 and miR-4500 inhibitors. All data were presented as the mean ± standard deviation and each was performed in triplicate. *P<0.05 vs. the control group. #P<0.05 vs. the si-STAT3 group. mir, microRNA; STAT3, signal transducer and activator of transcription 3; si-, small interfering RNA; MMP, matrix metalloproteinase.
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Availability of data and material

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and ZZ were major contributors in sample collection, performing the paper. HB, QZ, SZ, LZ and XZ participated in sample collection, performing the experiments and data interpretation. JZ produced the experimental design and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient and the present study was ethically approved by the Ethics Committee of the First People's Hospital of Changzhou (Changzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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