MicroRNA-19a functions as an oncogenic microRNA in non-small cell lung cancer by targeting the suppressor of cytokine signaling 1 and mediating STAT3 activation

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Abstract. MicroRNA-19a (miR-19a) has been found to be overexpressed in lung cancers. However, the underlying molecular mechanisms of miR-19a in tumorigenesis and the development of lung cancer remain poorly understood. In the present study, we aimed to delineate the role and mechanisms of action of miR-19a in non-small cell lung cancer (NSCLC). miR-19a was found to be overexpressed in both NSCLC tumor tissues and cell lines, as shown by RT-PCR. The enforced expression of miR-19a by transfection with miR-19a mimics significantly enhanced cell growth and viability, cell invasion and the migration of NSCLC cells, as shown by cell invasion and migration assays, and promoted the growth of xenograft tumors in a mouse xenograft tumor model. Conversely, the inhibition of miR-19a by transfection of the cells with miR-19a inhibitor displayed the opposite effects. More importantly, we found that miR-19a directly interacted with the 3’-untranslated region (3’-UTR) of the suppressor of cytokine signaling 1 (SOCS1) by dual-luciferase reporter assay. miR-19a was found to be capable of regulating the expression of SOCS1 in NSCLC cells. Thus, by modulating SOCS1 expression, miR-19a regulated the expression of the signal transducer and activator of transcription 3 (STAT3). Taken together, our data provide a possible underlying mechanism of action of miR-19a in the development of NSCLC and suggest that miR-19a may be a novel and promising target for therapeutic intervention in NSCLC.

Introduction

Among the many types of cancer, it is universally known that lung cancer has the highest morbidity and mortality worldwide (1). Clinical trials have demonstrated that approximately 80% of lung cancers are non-small cell lung cancer (NSCLC) with a fairly low survival rate (5-year survival rate <15%) (2,3). Nowadays, in spite of the fact that substantial progress has been made with traditional therapies, including combination regimens, patients with advanced NSCLC still have a poor prognosis (4-6). Therefore, the challenge in the treatment of NSCLC is to identify novel targets that may complement therapies.

Recently, microRNAs (miRNAs or miRs) have been implicated in various diseases (7). miRNAs with a length of 18-24 nucleotides modulate protein translation by directly binding the 3'-untranslated region (3'-UTR) of the target mRNA, thereby leading to mRNA destabilization and degradation (8,9). By modulating the protein expression of target genes, miRNAs are involved in the regulation of numerous cellular processes, including cell growth, proliferation, apoptosis, differentiation, migration and metabolism (10,11). Therefore, the dysregulation of miRNAs has been observed in a variety of cancer types (12,13). miRNAs not only play an important role in tumorigenesis and cancer treatment, but are also useful predictors for diagnosis and prognosis (14-16). Hence, cancer-related miRNAs may represent a novel and potential target for the therapeutic intervention of cancer.

It has been demonstrated that the miR-17-92 cluster located on chromosome 13q31.3 comprises a subset of oncogenic miRNAs that are extensively overexpressed in many types of cancer (17). Compelling evidence has indicated that miR-19a, belonging to the miR-17-92 cluster, is associated with the pathogenesis and development of many types of human cancer, including gastric cancer (18), cervical cancer (19) and colon cancer (20). The overexpression of miR-19a in the serum and tumor tissue of patients with NSCLC has been found to correlate with a worse prognosis (21). However, the role of miR-19a in regulating NSCLC is largely unknown.

In the present study, we aimed to delineate the role and the underlying mechanisms of action of miR-19a in the development of NSCLC. We found that miR-19a was not only overexpressed in tumor tissues of patients with NSCLC, but also in A549 and...
Materials and methods

Tissue samples, cell culture and animals. Fifteen pairs of lung cancer tissue samples and adjacent normal tissues were obtained from the Second Affiliated Hospital of Zhengzhou University, Zhengzhou, China after obtaining informed consent from the patients and approval from the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University. The average age of the patients was 44.08±3.05 years. Eleven of the specimens were lung squamous cell carcinoma and 4 of the specimens were lung adenocarcinoma. The surgical removal of the lung tumors was carried out prior to treatment with chemotherapy and radiotherapy. The human NSCLC cell lines, A549 and NCI-H157, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco-BRL, Rockville, MD, USA) and 1% penicillin/streptomycin. The human embryonic kidney cell line, HEK293T, and the immortalized keratinocyte cell line, HaCaT, were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM supplemented with 10% FCS. All cells were cultured under a humidified atmosphere containing 5% CO₂ at 37°C in an incubator. Female, 6-week-old BALB/c nude mice (25-30 g) purchased from the Experimental Animal Center of Zhengzhou University were raised under pathogen-free conditions with free access to water and food. The animal experimental procedures were approved and reviewed by the Institutional Animal Care and Use Committee of Zhengzhou University.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the tissue samples and cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and small RNA was extracted using mirVana kits (Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions. A total of 5 μg RNA was reverse-transcribed into complementary DNA using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) and the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Gene expression was analyzed according to the RT-PCR protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for SOCS1) and U6 snRNA (for miR-19a) were used as internal references and the relative gene expression was quantified using the 2^A(Ct) method.

MTT assay. The cells were seeded in 96-well culture plates at 1x10⁴ cells/well. When the cells reached 80% confluence, 50 nM of miR-19a mimics, miR-19a inhibitor or scrambled control (GenePharma, Ltd., Shanghai, China) were transfected into the cells. After 48 h of transfection, 20 μl/well MTT (5 mg/ml in PBS) were added followed by continuous culture for 4 h. Thereafter, 200 μl/well dimethylsulfoxide were added to dissolve the formazan crystals for 15 min. The absorbance at 490 nm was measured using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA). The experiments were performed in quintuplicate and repeated 3 times.

Cell invasion and migration assays. The potential of cell invasion and migration was measured using Transwell inserts, as previously described (23). To determine the cell invasion ability, 24-well plates with Transwell chambers and pre-coated Matrigel membrane filter (Neuro Probe Inc., Gaithersburg, MD, USA) were prepared. A total of 500 μl DMEM plus 10% FCS was added to the lower chamber and 2x10⁵ cells resuspended in 200 μl of serum-free DMEM were plated in the upper chamber of chemotaxis chambers. After 48 h of incubation at 37°C, the inserts were removed and were submerged in PBS to remove the unattached cells. The cells were then fixed with paraformaldehyde (4%) and stained with crystal violet (0.1%; Sigma-Aldrich, St. Louis, MO, USA). The invading cells were photographed (x20) and 5 random fields on each membrane were selected for cell number counting. Cell migration ability was detected according to a protocol similar to that for the cell invasion assay using a Matrigel-uncoated 24-well Transwell plate. These experiments were carried out in triplicate.

Dual-luciferase reporter assay. The putative binding sequences of SOCS1 3'-UTR for miR-19a were predicted by miRanda (http://www.microrna.org/), miRBase (http://www.mirbase.org/) and Targetscan (http://www.targetscan.org/). The 3'-UTR and mutated 3'-UTR constructs of SOCS1 were amplified and subcloned into a pGL3 luciferase promoter vector (Promega Corp., Madison, WI, USA) with XhoI and NotI restriction sites. The HEK293T cells were transfected with 50 ng of the pGL3 vector, pGL3-SOCS1-3'-UTR or pGL3-SOCS1-mutated 3'-UTR (Mut-SOCS1-3'-UTR), and miR-19a mimics, miR-19a inhibitor or scrambled control (50 nM) were then added using lipofectamine transfection reagent (Invitrogen). After 48 h of incubation, the cells were harvested and the luciferase activity was determined using the dual-luciferase reporter assay kit (Promega Corp.). The relative quantification was normalized to the luciferase activity in the control group.

Western blot analysis. A total of 20 μg proteins was isolated by electrophoresis on 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Non-specific bindings in the membrane were blocked by skimmed milk (2.5%) for 1 h at 37°C. The membrane was then incubated with primary antibodies in blocking buffer at 4°C overnight. The primary antibodies were as follows: anti-SOCS1 (sc-7006), anti-STAT3 (sc-8019) and anti-GAPDH (sc-20357) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-p-STAT3 (#9145) was purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). After washing with Tris-buffered saline and Tween-20 (TBST) 3 times, secondary antibody conjugated with horseradish peroxidase (bs-0293Gs; Bios, Beijing, China) was added.
followed by incubation for 4 h. Finally, the protein band was detected using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The protein gray intensity was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The fold changes in protein expression were presented after normalization to the control group.

Mouse xenograft assay. For this experiment, 2x10^6 cells suspended in 200 µl of PBS were injected subcutaneously into the flanks of nude mice. Approximately 40 days after cell inoculation, the mice were injected subcutaneously with sodium pentobarbital (40 mg/kg). The tumors were isolated and the total RNA and protein were extracted for analysis.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Significant comparisons were carried out by the two-tailed Student's t-test or one-way ANOVA followed by the Bonferroni post hoc test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

miR-19a is overexpressed in lung cancer tissues and NSCLC cells. To investigate the role of miR-19a in the tumorigenesis of NSCLC, we firstly identified the expression profile of miR-19a in tumor tissues and adjacent normal tissues by RT-PCR. The results revealed that the expression of miR-19a was markedly increased in the human NSCLC samples in comparison with the normal samples (Fig. 1A). Furthermore, the expression of miR-19a in the NSCLC cell lines, A549 and NCI-H157, was measured and the data indicated that miR-19a was significantly upregulated in the A549 and NCI-H157 cells in comparison with the control cells (HaCaT cells; Fig. 1B). These results suggest that miR-19a may be involved in the mediation of tumorigenesis and the development of NSCLC.

miR-19a enhances the cell growth and viability of NSCLC cells. To determine the effects of miR-19a on the growth of NSCLC cells, we transfected the A549 and NCI-H157 cells with miR-19a mimics or miR-19a inhibitor and detected cell growth and viability by MTT assay. The results revealed that transfection of the cells with miR-19a mimics significantly enhanced the growth and viability of the A549 and NCI-H157 cells (Fig. 2A). Conversely, transfection with miR-19a inhibitor markedly inhibited the growth and viability of the A549 and NCI-H157 cells (Fig 2B). These results suggest that miR-19a positively regulates the growth and viability of NSCLC cells.

miR-19a promotes the invasion and migration of NSCLC cells. To further explore the function of miR-19a in NSCLC....
cells, we determined the effects of miR-19a on the invasion and migration ability of A549 cells. Transfection with miR-19a significantly increased the cell invasion (Fig. 3A) and migration (Fig. 3B) ability of the A549 cells. By contrast, cell invasion (Fig 3C) and migration (Fig. 3D) were markedly reduced by transfection of the A549 cells with miR-19a inhibitor. These data indicate that miR-19a promotes the invasion and migration of NSCLC cells.

**SOCS1 is a direct target gene of miR-19a.** To elucidate the mechanisms of action of miR-19a in regulating the development of NSCLC, the candidate target genes of miR-19a were predicted by bioinformatics analysis. Of these genes, SOCS1, a critical tumor-associated gene (24), was found to have putative bindings sites within the 3’-UTR of miR-19a (Fig. 4A). To confirm the interaction of miR-19a with the 3’-UTR of SOCS1, we subcloned the 3’-UTR or mutated 3’-UTR of SOCS1 downstream of the luciferase reporter gene in the pGL3 plasmid. These vectors were co-transfected with miR-19a mimics into HEK293T cells. The results revealed that transfection with miR-19a mimics and pGL3-SOCS1-3’-UTR significantly inhibited luciferase activity compared with the controls, whereas the vector bearing the mutated 3’-UTR of SOCS1 was not affected by transfection with miR-19a mimics (Fig. 4B). These data suggest that miR-19a directly binds to the 3’-UTR of SOCS1.

**miR-19a regulates the mRNA and protein expression levels of SOCS1.** To determine whether miR-19a has an effect on endogenous SOCS1 expression, we examined the mRNA and protein expression levels of SOCS1 in A549 cells transfected with miR-19a mimics or miR-19a inhibitor. We found that transfection with miR-19a mimics and pGL3-SOCS1-3’-UTR significantly decreased the mRNA levels of SOCS1 (Fig. 5A) and decreased the protein levels of SOCS1 (Fig. 5B). By contrast, the inhibition of miR-19a markedly upregulated the mRNA (Fig. 5C) and protein levels of SOCS1 (Fig. 5D). These results suggest that miR-19a directly binds to the 3’-UTR of SOCS1.

**miR-19a mediates the activation of STAT3.** Previous studies have suggested that SOCS1 negatively regulates the activation of STAT3 (25-27). The constitutive activation of STAT3 has been observed in various types of cancer, including NSCLC (22). Therefore, considering the regulatory effect of miR-19a on SOCS1, we hypothesized that miR-19a may affect the activation of STAT3. To confirm this hypothesis, we examined the activation state of STAT3 in cells transfected with miR-19a mimics or miR-19a inhibitor. As expected, the phosphorylation of STAT3 was significantly increased following transfection.

Figure 3. Effects of miR-19a on cell invasion and migration. Detection of miR-19a on (A) invasion and (B) migration, or miR-19a inhibitor on (C) invasion and (D) migration of A549 cells. The number of cells was calculated by averaging 5 random fields. *p<0.05.

Figure 4. Suppressor of cytokine signaling 1 (SOCS1) is a direct target gene of miR-19a. (A) The predicted binding sequences for SOCS1 3’-untranslated region (UTR) with miR-19a. (B) Luciferase activity assay was performed to detect the interaction between SOCS1 3’-UTR and miR-19a. The wild-type or mutated SOCS1 3’-UTR was co-transfected with miR-19a mimics into HEK293T cells followed by incubation for 48 h. The luciferase activity was measured by dual-luciferase reporter assay. N=3, *p<0.05 and #p>0.05.
with miR-19a mimics (Fig. 6A), whereas transfection with miR-19a inhibitor markedly inhibited the phosphorylation of STAT3 (Fig. 6B). These results suggest that the activation of STAT3 is regulated by miR-19a.

**miR-19a promotes xenograft tumor growth.** To obtain further insight into the mechanisms of action of miR-19a in NSCLC, we inoculated A549 cells into nude mice and measured the effects of miR-19a expression on xenograft tumor growth. The intratumoral injection of lentiviral vector expressing miR-19a (LV-miR-19a) significantly increased tumor growth, whereas the injection of lentiviral vector expressing anti-miR-19a oligonucleotide (LV-anti-miR-19a) markedly decreased tumor growth (Fig. 7A). By analyzing the xenograft tumor tissues, we found that the injection of LV-miR-19a significantly inhibited SOCS1 expression and increased the phosphorylation of STAT3, whereas the injection of LV-anti-miR-19a exerted the opposite effects (Fig. 7B-D). These results suggest that miR-19a regulates tumor growth through the mediation of SOCS1 expression and STAT3 activation.
miR-19a, as an oncogenic miRNA, has been found to be dysregulated in various types of cancer. miR-19a has been found to be overexpressed in tissue specimens of esophageal squamous cell carcinoma, which is suggested as a potential unfavorable prognostic biomarker (28). Recently, it has been demonstrated that following serum therapy, miR-19a is significantly decreased in early breast cancer and high-risk patients are those with highly abundant serum levels of miR-19a (29). In lung cancer development, a high level of miR-19a has been observed in advanced NSCLC (30). Accordingly, high serum levels of miR-19a have been found to be an independent prognostic factor for worse survival in patients with NSCLC (21). In line with these previous findings, in this study, we provide evidence that miR-19a is overexpressed in both NSCLC tumor tissues and cell lines. The aberrant high expression of serum miR-19a has been found to be a predictor of resistance following chemotherapy (31). Our results further revealed that miR-19a was an oncogenic gene, suggesting that miR-19a may not only be a diagnostic and prognostic marker, but also a potential target for the development of lung cancer therapeutics.

The dysregulated expression profile of miR-19a indicates that miR-19a may participate in the regulation of tumorigenesis. The inhibition of miR-19a has been shown to lead to a decrease in tumor cell proliferation and brain allografts in vivo of medulloblastoma and to prolong the survival of mice (32). Li et al previously demonstrated that curcumin is capable of downregulating the miR-19a expression induced by bisphenol A in early breast cancer and exhibits antitumor effects through the modulation of the phosphatase and tensin homolog (PTEN)/AKT/p53 axis (33). miR-19a, along with miR-19b, promotes the metastasis of gastric cancer cells by directly targeting and inhibiting the tumor suppressor, MAX dimerization protein 1 (MXD1) (18). In laryngeal squamous cell carcinoma, miR-19a overexpression has been suggested to correlate with reduced overall survival and the inhibition of miR-19a has been shown to increase cell apoptosis and decrease cell proliferation (34). By targeting PTEN, miR-19a promotes multidrug resistance in gastric cancer cells (35). Therefore, miR-19a functions as an oncogene in various types of cancer by regulating different target genes. To the best of our knowledge, for the first time, in the present study, we investigated the role of miR-19a in the development of NSCLC. We found that transfection with miR-19a mimics markedly enhanced the growth, cell invasion and migration of NSCLC cell lines. Our findings are consistent with those of Xu et al, who demonstrated that miR-19a regulates cell proliferation and the invasion of cervical carcinoma cells by targeting cullin 5 (19). However, in this study, using dual-luciferase reporter assay, we found that SOCS1, a critical tumor-associated gene (24), was a direct target gene of miR-19a in NSCLC. In NSCLC cells, we further demonstrated that miR-19a regulated the mRNA and protein expression of SOCS1. These data suggest that miR-19a plays an important role in NSCLC by direct targeting and regulating SOCS1 expression.

SOCS1 functions as a negative regulator of inflammation (36,37) and plays critical roles in cells and animals (38-40).
The role of SOCS1 in the regulation of cancer has been studied extensively (24,41). The inhibited expression profiles of SOCS1 have been demonstrated in numerous types of cancer, including liver cancer (42), prostate cancer (43) and pancreatic cancer (44). The downregulation of SOCS1 has been found to be associated with prolonged JAK/STAT signaling pathway activity (45,46). It has been suggested that SOCS1 is able to interact with JAK through the SH2 domain, leading to STAT inactivation. The constitutive activation of JAK2/STAT3 has been shown in various solid tumors, including NSCLC (22,47,48). The enforced expression of SOCS1 exerts an anti-proliferative effect through the inactivation of STAT3 and the p38 MAPK pathway in gastric cancer cells (49). The oncolytic adenovirus-mediated SOCS1 expression has been shown to exert an antitumor effect through the inhibition of STAT3 phosphorylation in hepatocellular carcinoma cells (27). Shikonin has been reported to inhibit the interleukin (IL)-17-induced activation of STAT3 by upregulating SOCS1 (50). Huang et al demonstrated that miR-155 promotes pancreatic cancer cell invasion and migration by inhibiting SOCS1 expression and activating STAT3 signals (25). In the present study, we demonstrated that miR-19a suppressed the downstream target gene, SOCS1, and increased the phosphorylation of STAT3. Therefore, the miR-19a-induced increase in the activation of STAT3 may occur through the inhibition of SOCS1 expression.

Shimada et al demonstrated that SOCS1 overexpression exerts a more potent inhibitory effect on STAT3 activation than JAK inhibitors in NSCLC cells (51), suggesting a significant antitumor role of SOCS1 in NSCLC. In this study, we demonstrated that miR-19a, which is highly overexpressed in NSCLC, directly targeted and inhibited SOCS1 expression. The inhibition of miR-19a significantly upregulated SOCS1 expression and exerted a marked antitumor effect in vitro and in vivo. Taken together, our data suggest that miR-19 functions as an oncogenic miRNA and targeting miR-19a may provide novel strategies for the development of anti-NSCLC therapeutics.

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References