miR-506 functions as a tumor suppressor in glioma by targeting STAT3

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Abstract. MicroRNA-506 (miR-506) has been reported to act as a tumor suppressive or an oncogenic miRNA in different types of tumors. However, the roles and underlying molecular mechanism of miR-506 in glioma remain unclear. In the present study, we performed quantitative PCR to investigate the level of miR-506 in 36 pairs of glioma tumor and matched adjacent tissues, and found that miR-506 was downregulated in the glioma tumors compared to the expression in the adjacent normal tissues. Furthermore, a functional assay found that ectopic expression of miR-506 in glioma cells markedly suppressed cell proliferation, colony formation, migration and invasion, and suppressed tumor growth in vivo. Moreover, signal transducer and activator of transcription 3 (STAT3) was identified as a direct target of miR-506. Western blot assay showed that overexpression of miR-506 not only induced changes in STAT3 expression but also altered expression of its downstream genes, including, Bcl2, cyclin D1 and matrix metalloproteinase 2 (MMP-2), in the human glioma cells. In addition, STAT3 mRNA expression was increased in the glioma tissues, and was inversely correlated with miR-506. Importantly, overexpression of STAT3 in glioma cells attenuated the suppressive effects of miR-506 on cell proliferation, colony formation, migration and invasion. These results showed that miR-506 functions as a tumor suppressor in glioma by targeting STAT3, suggesting that miR-506 may serve as a potential target in the treatment of human glioma.

Introduction

Glioma is the most common primary tumor of the central nervous system and is associated with high morbidity and mortality. Glioma accounts for ~80% of malignant brain tumors (1,2). Despite therapeutic advances, the median survival duration of patients with glioblastoma multiforme (GBM), the most aggressive type of malignant glioma, has not significantly improved due to difficulties in complete resection and the low sensitivity to radiotherapy and chemotherapeutic agents (3-5). Thus, it is quite urgent to understand the molecular mechanisms by which glioma initiates, progresses, invades and recurs in order to develop effective prognostic biomarkers and novel therapies.

MicroRNAs (miRNAs) are small (19-24 nucleotides), single-stranded, non-coding RNA molecules (18-24) that usually lead to gene silencing by binding to complementary sequences in the three prime untranslated regions (3'UTRs) of target messenger RNA (mRNA) transcripts (6-8). miRNAs are involved in various biological processes, such as cell division, cell cycle, differentiation, proliferation development and apoptosis (9-11). Growing evidence shows that miRNAs are involved in the progression and development of human cancers, either as oncogenes or tumor suppressors, providing new insight into the diagnosis, prognosis and therapy for various types of tumors (12,13).

miR-506, a recently discovered miRNA, has been reported to function as a tumor suppressor in human cancers including cervical cancer (14), breast cancer (15), epithelial ovarian cancer (16), oral squamous cell carcinoma (17), and gastric cancer (18). However, the role of miR-506 in glioma and the mechanisms underlying glioma carcinogenesis remain unclear. Therefore, the aims of the present study were to investigate the role of miR-506 and the underlying molecular mechanisms in glioma.

Materials and methods

Clinical glioma samples. Primary glioma tissues and adjacent non-tumor tissues were obtained from 36 adult patients who underwent glioma resection at the Department of Neurosurgery, the First Hospital of Jilin University (Changchun, China). None of the patients had received chemotherapy, immunotherapy and radiotherapy prior to surgery. All samples were immediately frozen in liquid nitrogen and stored at -80˚C until use. All patients gave written informed consent before surgery. The study protocol and consent procedures were approved by the Ethics Committee of Jilin University (Changchun, China).

Cell lines and culture. Primary normal human astrocytes (NHA) and 4 human glioma cell lines (U251, U87, U118 and
LN18) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA), 100 U/ml penicillin or 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from the cultured cells and frozen tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For miR-506 expression, total RNA was reversely transcribed into cDNA using One Step PrimeScript miRNA cDNA Synthesis kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Then the expression levels of miR-506 were quantified using TaqMan miRNA assay kits under the ABI 7900 Fast system (both from Applied Biosystems, Foster City, CA, USA). To quantify STAT3, total RNA was reversely transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China). The expression levels of STAT3 were quantified by Real-Time PCR Mixture reagent (Takara) under the ABI 7900 Fast system. The primers for STAT3 mRNA were: forward, 5'-GAA GAATCCAAACGGGCA-3' and reverse, 5'-TCACAATCA GGGAAGCAT-3'. U6 and GAPDH were used as internal controls for miRNAs and mRNAs, respectively. Relative expression was calculated using the 2-ΔΔCT method.

Cell transfection. The miR-506 mimic (miR-506) and corresponding miRNA negative control (miR-NC) were purchased form GenePharma Co., Ltd. (Shanghai, China). The STAT3 overexpression plasmid was designed and synthesized by Ribobio Co. (Guangzhou, China). These molecular products were transfected into U87 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were determined in every experiment at 48 h after transfection.

Cell proliferation and colony formation assay. Cell proliferation was measured by MTT assay. In briefly, 2x10³ transfected cells were seeded into 96-well plates and cultured for 24-72 h. After incubation at 37°C for 4 h, followed by removal of the MTT solution, 150 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. Optical density (OD) was detected at a wavelength of 570 nm. All experiments were performed in triplicate.

For the colony formation assay, 1,000 transfected cells were seeded in 6-well plates and cultured for 14 days at 37°C under 5% CO₂. Then the colonies were fixed with 75% ethanol for 10 min, dried and stained with 0.1% crystal violet solution for 10 min. Then images were captured of the colonies, and the number of colonies was counted under a light microscope (Olympus, Tokyo, Japan).

Cell cycle assay. Cells cycle analysis was performed on U87 cells 48 h after transfection. The transfected cells were harvested, washed, fixed in ice-cold 75% ethanol and stored at -20°C for 12 h. The cells were resuspended in PBS containing 25 mg/ml propidium iodide (PI), 0.1% Triton X-100, and 10 mg/ml RNase and incubated at 4°C for 30 min in the dark. The cells were then analyzed by fluorescence-activated cell sorting (FACS; BD Biosciences, Mansfield, MA, USA).

Wound-healing assay. The transfected cells (2x10⁴) were seeded into 24-well culture plates and cultured for 24 h at 37°C under 5% CO₂. Then an artificial homogeneous wound was created onto the monolayer with a 20-µl sterile plastic micropipette tip. After wounding, the debris was removed by washing the cells with PBS. To visualize the migrating cells and wound healing, images were captured at 0 and 24 h after wounding.

Invasion assays. The transfected cells (2x10⁴) were placed into Transwell chambers (8.0-µm pore size; Corning Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, Bedford, MA, USA) in serum-free medium. DMEM containing 20% FBS in the lower chamber served as the chemoattractant. After the cells were incubated for 48 h at 37°C with 5% CO₂, the cells that had invaded through the membrane were fixed in 90% alcohol and stained with 0.1% crystal violet for 5 min and then photographed. The number of invaded cells was counted in five randomly selected fields under a light microscope (x200; Olympus).

Vector construction and luciferase assays. The complimentary sequence of STAT3 3’UTR for miR-506 (STAT3-Wt) and mutated 3’UTR sequence (STAT3-Mut) were synthesized and inserted into the pGL3-control vector (Ambion, Austin, TX, USA) at the Nhel and Xhol restriction sites. For the luciferase assays, 1x10⁴ cells were plated in 24-well plates and cultured for 24 h. Then the cells were co-transfected with 100 ng of STAT3-Wt or STAT3-Mut reporter plasmid, and 100 nM of miR-506 mimic or miR-NC using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 h after transfection, both firefly and Renilla luciferase activities in the cell lysates were determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). Renilla luciferase was used for normalization.

Western blotting. Cells were harvested and lysed in ice-cold RIPA buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Concentrations of total cellular protein were quantified using the BCA protein assay kit (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China) according to the manufacturer's instructions. Equal amounts of protein lysates (20 µg each lane) were separated by 8-12% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membrane was incubated at 4°C overnight with the following primary antibodies: anti-STAT3 (1:1,000), anti-cyclin D1 (1:2,000) and anti-Bcl-2 (1:1,000) (all from Santa Cruz Biotechnology), anti-MMP-2 (1:1,000), anti-cyclin D1 (1:2,000), anti-Bcl-2 (1:1,000), anti-MMP-2 (1:1,000) and anti-GAPDH (1:5,000; Santa Cruz Biotechnology) and anti-Bcl-2 (1:1,000), anti-cyclin D1 (1:2,000), anti-Bcl-2 (1:1,000). The membranes were incubated at 4°C overnight with the following primary antibodies: anti-STAT3 (1:1,000), anti-cyclin D1 (1:2,000), and anti-Bcl-2 (1:1,000) (all from Santa Cruz Biotechnology), anti-MMP-2 (1:1,000; Cell Signaling Technology) and anti-GAPDH (1:5,000; Santa Cruz Biotechnology), followed by the corresponding secondary antibody labeled with HRP and detected by enhanced chemiluminescence (ECL; Cell Signaling Technology). Protein quantity was detected by GAPDH as a loading control.

In vivo tumor model. Twenty female BALB/c mice (4-5 weeks of age) were obtained from the Experiments Animal Center.
of Changchun Biological Institute (Changchun, China), and maintained under specific pathogen-free (SPF) conditions. All procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. All animal protocols were approved by the Institutional Animal Care and Use Committee of Jilin University (Changchun, China).

U87 cells (2x10⁶) stably expressing miR-506 or miR-NC were directly injected subcutaneously into the flanks of nude mice (n=10), respectively. Tumor volume (TV) was determined by caliper every week according to the formula: TV (mm³) = 1/2 x width² x length. After 5 weeks of inoculation, all mice were sacrificed and the tissues were removed and weighed. Part of the tumor tissues were harvested for analysis of the expression of miR-506 and STAT3.

Statistical analysis. Data from at least three independent experiments are expressed as the mean ± SD (standard deviation). The differences between two groups were analyzed using the two-sided Student’s t-test, and analysis of more than two groups was performed using one-way ANOVA followed by a Tukey’s post hoc test. All data were analyzed using the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) and the SPSS 19.0 software (SPSS, Chicago, IL, USA). P<0.05 was used to indicate a statistically significant difference.

Results

**miR-506 expression is decreased in glioma tissue samples and cell lines.** The expression of miR-506 was detected in 36 pairs of human glioma and adjacent normal tissues by real-time quantitative RT-PCR (qRT-PCR). As shown in Fig. 1A, we found that the relative expression levels of miR-506 were significantly lower in the glioma tissues than levels in the adjacent normal tissues (P<0.01). In addition to glioma tissues, endogenous expression of miR-506 was detected in four human glioma cell lines (U251, U87, U118 and LN18) and normal human astrocytes (NHAs). It was found that the miR-506 expression in the four glioma cell lines was significantly reduced relative to that in the NHAs (Fig. 1B). The U87 cell line, which possessed the lowest level of miR-506 expression among the four cell lines, was therefore selected for the subsequent studies.

**miR-506 inhibits the cell proliferation and colony formation of glioma cells.** The decreased expression of miR-506 in glioma tissues and cell lines inspired us to hypothesize that miR-506 is a tumor suppressor in glioma. To test the role of miR-506 in glioma growth, miR-506 or miR-NC was transfected into U87 cells and cultured for 48 h, and then miR-506 expression was determined by qRT-PCR. Our results showed that the intracellular level of miR-506 was higher in the U87 cells transfected with the miR-506 mimic compared with the levels in cells transfected with miR-NC (Fig. 2A). Meanwhile, cell proliferation and colony formation were determined in the U87 cells after transfection of miR-506 or miR-NC. We found that overexpression of miR-506 significantly inhibited cell proliferation (Fig. 2B) and colony formation (Fig. 2C) in the U87 cells (P<0.05). As proliferation is directly connected to cell cycle distribution, the effect of miR-506 on cell cycle progression was also analyzed in the U87 cells. As expected, the percentage of G0/G1 phase cells was increased, and the percentage of S phase cells was decreased in the U87 cells transfected with the miR-506 mimic compared to the percentage in the cells transfected with miR-NC (P<0.05, Fig. 2D). These results suggest that miR-506 inhibits glioma cell growth in vitro.

**miR-506 inhibits the cell migration and invasion of glioma cells.** To reveal the biological role of miR-506 on migration and invasion, we used a wound healing and invasion chamber assays, respectively. It was found that overexpression of miR-506 significantly inhibited the migration (Fig. 3A) and invasion (Fig. 3B) capacities in the U87 cells.

**STAT3 is a direct target of miR-506.** To understand how miR-506 regulates cell growth and metastasis, we used
Figure 2. Overexpression of miR-506 inhibits the proliferation and colony formation of glioma cells. (A) qRT-PCR was used to determine the miR-506 expression in U87 cells transfected with the miR-506 mimic or miR-NC. (B) Cell proliferation was determined by MTT assay in U87 cells transfected with the miR-506 mimic or miR-NC. (C) Cell colony formation ability was determined in the U87 cells transfected with the miR-506 mimic or miR-NC. (D) Cell cycle arrest was determined in U87 cells transfected with the miR-506 mimic or miR-NC. **P<0.01 vs. miR-NC.

Figure 3. Overexpression of miR-506 inhibits the migration and invasion of glioma cells. (A) Cell migration was determined by a wound-healing assay in the U87 cells transfected with the miR-506 mimic or miR-NC. (B) Cell invasion was determined in the U87 cells transfected with the miR-506 mimic or miR-NC. **P<0.01 vs. miR-NC.
two algorithms (TargetScan and miRanda) to help identify miR-506 target genes. STAT3 was selected as the potential target of miR-506, since STAT3 has been found to be involved in the tumorigenesis and metastasis of glioma (19,20). To further confirm whether STAT3 is a direct target of miR-506, a human STAT3 3'UTR fragment containing the binding sites of miR-506 or the mutant sites (Fig. 4A) were cloned into the pGL3 vector, and the miR-506 mimic or miR-NC were co-transfected into U87 cells for luciferase activity. It was found that overexpression of miR-506 markedly suppressed the luciferase activity of the STAT3-Wt 3'UTR, without having an effect on STAT3-Mut 3'UTR in the U87 cells (Fig. 4B). We further found that the mRNA and protein levels of STAT3 were decreased in the U87 cells transfected with miR-506 compared with the miR-NC group (Fig. 4C and D). In addition, we found that overexpression of miR-506 inhibited STAT3 downstream protein expression, such as cyclin D1, Bcl-2 and MMP-2 (Fig. 4D).

miR-506 expression is inversely correlated with STAT3 expression in glioma tissues. We also examined the expression of STAT3 in glioma specimens and the corresponding non-cancerous tissues from 36 glioma patients by qRT-PCR. It was found that the STAT3 mRNA expression level was increased in the glioma tissues compared to that in the paired non-cancerous tissues (Fig. 5A), and was negatively correlated with miR-506 (Fig. 5B; r=-0.632, P<0.001).

miR-506 suppresses glioma progression by targeting STAT3. We further aimed to ascertain whether overexpression of STAT3 could reverse the suppressive effect of miR-506. U87 cells were transfected with the miR-506 mimic or miR-NC, followed by transfection with the STAT3 overexpression plasmids. The overexpression of STAT3 at the mRNA level (Fig. 6A) and protein level (Fig. 6B) was validated by qRT-PCR and western blotting assay, respectively. In addition, our results revealed that overexpression of STAT3 in the U87 cells attenuated the effect of miR-506 on cell proliferation, colony formation, migration and invasion (Fig. 6C-F). Taken together, these results indicate that the tumor-suppressor role of miR-506 is mediated by targeting STAT3.

miR-506 suppresses glioma tumorigenicity in vivo. The in vitro study indicated that miR-506 inhibits glioma cell growth, we therefore investigated whether miR-506 suppresses tumor growth in vivo. The human U87 cells stably expressing miR-506 or miR-NC were implanted subcutaneously into nude mice to allow tumor formation. At 5 weeks post-injection, the mice were sacrificed, and tumor tissues were extracted. Our results showed that miR-506-expressing U87 tumors were significantly smaller than that of miR-NC-expressing U87 tumors (Fig. 7A). The average volume and weight of the miR-506-expressing U87 tumors were significantly decreased compared with the volume and weight of the miR-NC-expressing U87 tumors (both P<0.01, Fig. 7B and C). Furthermore, the expression of miR-506 and STAT3 in xenograft tumor tissues was determined. It was found that miR-506 expression was upregulated (Fig. 7D), while STAT3 expression at the mRNA and protein level was decreased in the miR-506-expressing U87 tumors (Fig. 7E and F).
results indicate that miR-506 suppresses glioma growth in vivo by targeting STAT3.

**Discussion**

Malignant gliomas are the most common primary tumors of the central nervous system and are associated with high morbidity and mortality (1,2). To date, no effective treatment method has been found for the recurrence of malignant gliomas. Recently, accumulating evidence indicates that the aberrant expression of miRNAs contributes to glioma tumorigenesis and development by inhibiting the expression of their target genes, proposed as molecular biomarkers for prediction and prognosis of glioma, and as novel targets for glioma treatment (19,20). Therefore, there is an urgent need to search for specific miRNAs involved in tumorigenesis for the diagnosis and therapy of patients with glioma. In the present study, we report for the first time that miR-506 is significantly downregulated in glioma clinical specimens and cell lines. The overexpression of miR-506 in glioma cells inhibited...
proliferation, colony formation, migration and invasion of glioma cells in vitro, and suppressed glioma tumor growth in vivo. STAT3 was identified as a new direct and functional target of miR-506 by using dual-luciferase assay, and its expression at the mRNA and protein level was downregulated after transfection with the miR-506 mimic in glioma cells by qPCR and western blot analysis. We also found that STAT3 expression was upregulated in glioma tissues, and was negatively correlated with miR-506. In addition, overexpression of STAT3 partially rescued the suppressive effect of miR-506. These findings suggest that miR-506 is a novel molecular therapeutic target for glioma.

miR-506, located on chromosome X, has been reported to be involved in diverse biological behaviors depending on different target genes. It has been shown that miR-506 expression is downregulated in several types of cancers such as gastric (18), cervical (14), ovarian (21) and lung cancer (22), suggesting that miR-506 plays an important role in tumorigenesis and tumor progression. Yang et al reported that miR-506 is downregulated in clear cell renal cell carcinoma and inhibits cell growth and metastasis via targeting forkhead box Q1 (FLOT1) (23). Sun et al found that miR-506 regulates both E-cadherin and vimentin/N-cadherin in the suppression of epithelial-mesenchymal transition (EMT) and metastasis in ovarian cancer (24). Arora et al showed that expression of miR-506 was decreased in breast cancer tissues and cell lines, and that miR-506 regulated breast cancer EMT and invasion by targeting vimentin, Snai2 and CD151 (25). These studies suggest that miR-506 potentially functions as a tumor suppressor in these cancers. In contrast, in hydroxycamptothecin-resistant human colon cancer and melanoma cells (26,27), miR-506 acts as an oncogene. These controversial findings suggest that miR-506 may have different roles depending on the cancer type. To investigate the potential role of miR-506 in glioma, we analyzed the expression of miR-506 in 36 glioma tumors and their paired non-cancerous tissues by qPCR. Our results showed that miR-506 was significantly downregulated in the glioma clinical specimens and cell lines. Functional assays showed that miR-506 inhibited glioma growth in vitro and in vivo partially by targeting STAT3. These results suggest that miR-506 may function as a tumor suppressor miRNA in glioma.

It is well known that miRNAs usually exert their biological functions by regulating target gene expression (28). In this study, we used two bioinformatic algorithms to predict gene targets for miR-506, and found that the signal transducer and activator of transcription 3 (STAT3) contains a highly conserved miR-506 binding site on the 3'UTR. Luciferase assay further confirmed that STAT3 is a direct target of miR-506 in glioma cells. STAT3, an important member of the STAT family, has been showed to be upregulated in a wide variety of human tumors including glioma (29). Aberrantly active STAT3 promotes cell proliferation, migration and invasion, as well as inhibition of apoptosis and aberrant cell cycle progression via incessant induction of pro-growth genes, such as cyclin D1, c-Myc, survivin, Bcl-xL, Bcl-2, Mcl-1, VEGF, MMP-2 and MMP-9 (30-35). Here we showed that overexpression of miR-506 decreased STAT3 and expression of its downstream proteins (Bcl-2, cyclin D1, MMP-2). In addition, we confirmed that STAT3 expression is upregulated in glioma tissues and is negatively correlated with miR-506. Of note, overexpression of STAT3 partially rescued the suppressive
effect of miR-506 in glioma cells. These results showed that miR-506 exerted a suppressive effect on glioma growth and metastasis partially by targeting STAT3.

In summary, to the best of our knowledge, our study provides initial evidence that the expression of miR-506 is downregulated in glioma tissues and cell lines, and functions as a novel tumor suppressor to inhibit the proliferation, colony formation, migration and invasion of glioma cells in vitro, and suppresses glioma tumor growth in vivo by targeting STAT3. These findings suggest that miR-506 may be a novel molecular therapeutic target for the treatment of glioma.

References


