MicroRNA-186 targets IGF-1R and exerts tumor-suppressing functions in glioma

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Received July 28, 2016; Accepted June 26, 2017

DOI: 10.3892/mmr.2017.7586

Abstract. Glioma is the most common malignant brain tumor in adults and represents one of the most aggressive and life-threatening types of cancer in humans. Increasing studies have revealed that microRNAs are abnormally expressed in various types of human cancer, and have oncogenic or tumor suppressive roles, which depend primarily on the type of cancer. The present study aimed to investigate the expression level and effects of microRNA-186 (miR-186) on glioma, and its underlying molecular mechanism. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to detect the expression of miR-186 in glioma tissues and cell lines. Cell proliferation and invasion were assessed using MTT and cell invasion assays, respectively. Bioinformatics analysis and a luciferase reporter assay were performed to identify insulin-like growth factor 1 receptor (IGF-1R) as a novel target gene of miR-186. The mRNA expression level of IGF-1R was also measured using RT-qPCR analysis. The association between miR-186 and the expression of IGF-1R was evaluated using Spearman's correlation analysis. Furthermore, the regulatory effects of miR-186 on the mRNA and protein expression of IGF-1R were determined using RT-qPCR and western blot analyses. Finally, the biological effects of the underexpression IGF-1R on glioma cells were investigated. The results showed that miR-186 was significantly downregulated in glioma tissues and cell lines. Inducing the expression of miR-186 suppressed glioma cell proliferation and invasion. IGF-1R was confirmed as a direct target gene of miR-186. In addition, the mRNA expression of IGF-1R was upregulated and inversely correlated with that of miR-186 in glioma tissues. The effects of IGF-1R-knockdown on glioma cell proliferation and invasion were similar to the effects induced by the overexpression of miR-186. These findings demonstrated that miR-186 acted as a tumor suppressor by targeting IGF-1R in glioma, suggesting miR-186 may be a potential therapeutic target for the treatment of this disease.

Introduction

Glioma is the most common malignant brain tumor in adults and represents one of the most aggressive and life-threatening types of human cancer (1). The morbidity rate of glioma is 3-8,000,000/10,000,000 individuals each year worldwide (2). Similar to other tumors, glioma primarily results from multiple genetic risk factors, poor lifestyle choices, infection and carcinogenic environmental factors, including biochemials in the environment, ionizing radiation, nitrous compounds and air pollution (3). According to the 2007 World Health Organization classification, glioma can be classified into three groups: Astrocytomas, which are well-differentiated, anaplastic astrocytomas, and glioblastoma multiforme, which is the most aggressive form (4). There has been substantial progress in early diagnosis and multimodal treatments for glioma, including surgery, local irradiation and conventional chemotherapy; however, there has been no marked improvement in the survival rates for patients with this disease (5). The high rates of recurrence and ineffective therapeutic strategies contribute to the poor prognosis of patients with glioma (6). Therefore, it is imperative to investigate the molecular mechanism involved in the occurrence and development of glioma, and identify novel effective therapeutic targets for the treatment of glioma.

MicroRNAs (miRNAs) are a group of conserved, endogenous, non-protein-coding, single stranded, short RNA molecules, which have a length of 17-27 nucleotides. miRNAs can negatively regulate gene expression through binding to complementary sequences in the 3'untranslated regions (3'UTRs) of their target genes, resulting in their translational regulation and/or direct cleavage (7,8). miRNAs are widely expressed in animal and plant cells, and are involved in several biological processes, including cell proliferation, cell cycle, apoptosis, differentiation, metastasis, angiogenesis and metabolism (9-11). Increasing studies have revealed that miRNAs are dysregulated in various types of human cancer, and exert oncogenic or tumor suppressive roles, which primarily depend
on the type of cancer (12-14). For example, miRNA (miR)-211 is upregulated in non-small cell lung cancer, and acts as an oncogene through enhanced cell proliferation, colony formation and invasion (15). By contrast, the expression of miR-211 is lower in gastric cancer tissues. The ectopic expression of miR-211 has also been shown to suppress the progression of gastric cancer (16). Therefore, miRNAs offer potential for investigation as specific diagnostic and prognostic markers, and as therapeutic targets in oncology.

The aim of the present study was to examine the expression, functional roles and underlying mechanism of miR-186 in glioma. This may provide a novel basis for development of the mechanism of glioma.

Materials and methods

Tissue specimens and cell lines. The present study was approved by the Ethical Committee of the Affiliated Hua’ian First Hospital of Nanjing Medical University (Hua’ian, China). Fresh glioma tissues (n=16) and adjacent normal tissues (n=16) were collected from patients with glioma during surgery at the Department of Neurosurgery, the Affiliated Hua’ian First Hospital of Nanjing Medical University, between June 2014 and January 2016. None of these patients had been treated with chemotherapy or radiotherapy prior to surgery. All fresh tissues were frozen in liquid nitrogen and stored at -80°C until use.

A total of five glioma cell lines (U251, U87, U373, 144 and A172) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The normal human astrocyte cell line (NAH) was purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humid atmosphere containing 5% CO₂ at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from tissues and cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The concentration and purity of total RNA was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RT process was performed using a TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using a TaqMan microRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with U6 as an internal control for determining the expression of miR-186. The reaction system for qPCR contained 1 μl TaqMan® Small RNA assay (20X), 1.33 μl cDNA, 10 μl TaqMan® Universal PCR Master mix II (2X) and 7.67 μl nuclease-free water. The thermocycling conditions for qPCR were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec. To determine the mRNA expression of IGF-1R, RT was performed using the M-MLV Reverse Transcription system (Promega Corporation, Madison, WI, USA). SYBR-Green I mix (Takara Biotechnology Co., Ltd., Dalian, China) was then used to detect the mRNA expression of IGF-1R with GAPDH as the internal control. This reaction for qPCR included 2 μl cDNA (100 ng), 2 μl forward primer, 2 μl reverse primer, 10 μl SYBR-Green PCR Master mix and 4 μl ddH₂O. The thermocycling conditions for qPCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The RT-qPCR analysis was performed using an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative mRNA expression of miR-186 and IGF-1R were quantified using the 2^ΔΔCq method (17).

Cell transfection. To examine the functions of miR-186 in glioma, miR-186 mimics and miRNA negative control (miR-NC) were purchased from GenePharma (Shanghai, China). The miR-186 mimics sequence was 5'-AUUUCUAGGGUCUCAUAUAGCGU-3' and the miR-NC sequence was 5'-UUCUCCGAACUGUGACGU TT-3'. Two small interfering RNAs (siRNAs), targeting IGF-1R (si-IGF-1R) and negative control (si-NC), were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The si-IGF-1R sequence was 5'-CCA CGT GCA GUC ACG UTT-3'. Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect oligonucleotides.

MTT assay. The cells were seeded into 96-well plates at a density of 3x10⁴ per well. Following incubation for 6-8 h, the cells were transfected with the miRNA mimics or siRNAs. The transfected cells were then incubated at 37°C in a humid atmosphere containing 5% CO₂ for 24, 48, 72 and 96 h. At each time point, 10 μl MTT solution (5 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to each well and incubated at 37°C for 4 h. Following incubation, the medium containing MTT solution was removed and 150 μl DMSO (Sigma-Aldrich; Merck Millipore) was added to each well. The absorbance of solution was then measured at 490 nm on a microplate reader.

Cell invasion assay. Transwell chambers (8-μm; Corning Costar, Cambridge, MA, USA) and pre-coated Matrigel (BD Biosciences, San Jose, CA, USA) were used to perform cell invasion assays. Briefly, 4x10⁴ of the transfected cells were resuspended in 100 μl FBS-free culture medium and seeded into the upper chambers, and 500 μl culture medium containing 20% FBS was added to the lower chambers. Following incubation for 48 h, any cells that had not invaded through the pores on the polycarbonate membranes, were carefully removed using a cotton swab. The invaded cells were fixed with 100% methanol, stained with 0.5% crystal violet and then counted under a light microscope (CKX41; Olympus Corporation, Tokyo, Japan; magnification, x200).

Target prediction. The potential target genes of miR-186 were analyzed using bioinformatics analysis with multiple target prediction algorithms, including TargetScan 7.1 (www.targetscan.org/vert_71/), miRanda (www.microrna.org/microrna/home.do) and PicTar (www.pictar.mdc-berlin.de/).

Luciferase reporter assay. The luciferase reporter plasmids, pGL3-Wt-IGF-1R-3'UTR and pGL3-Mut-IGF-1R-3'UTR, were
miR-186 is downregulated in glioma tissues and cell lines. To investigate the association between miR-186 and glioma, RT-qPCR analysis was performed to examine the expression of miR-186 in glioma tissues. As shown in Fig. 1A, the expression of miR-186 was downregulated in the glioma tissues, compared with that in the adjacent normal tissues. The expression levels of miR-186 in glioma cell lines were also detected, and the results of the RT-qPCR analysis revealed that the expression levels of miR-186 were lower in the glioma cell lines, compared with that in the NHA cell line (P<0.05; Fig. 1B). As the expression of miR-186 was lowest in the U251 and U87 cells, these two cell lines were selected for use in the subsequent experiments.

miR-186 suppresses the proliferation and invasion of U251 and U87 cells. To assess the functional roles of miR-186 in glioma, miR-186 mimics or miR-NC were injected into U251 and U87 cells. The transfection efficiency was evaluated using MTT and cell invasion assays, respectively. The results of the MTT assay showed that miR-186 reduced the proliferation of U251 and U87 cells (P<0.05; Fig. 2A). Furthermore, the expression of miR-186 was significantly higher in the U251 and U87 cells following transfection with miR-186 mimics (P<0.05; Fig. 2A). The possible effects of the overexpression of miR-186 on the proliferation and invasion of glioma cells were determined using MTT and cell invasion assays, respectively. The results of the MTT assay showed that miR-186 reduced the proliferation of U251 and U87 cells, compared with proliferation in the miR-NC groups (P<0.05; Fig. 2B). Furthermore, the overexpression of miR-186 significantly reduced the invasive ability of the U251 and U87 cells (P<0.05; Fig. 2C). These results suggested that miR-186 acted as a tumor suppressor in the tumorigenesis and progression of glioma.

IGF-1R is a direct target of miR-186. To investigate the molecular mechanism underlying the action of miR-186 in glioma, bioinformatics analysis was performed to determine the potential target genes of miR-186. The analysis found that the 'seed sequence' of miR-186 matched the 3’UTR of the IGF-1R (Fig. 3A). The correlation between the expression of miR-186 and the IGF-1R in glioma tissues was then examined. As shown
in Fig. 3B, the mRNA expression of IGF-1R was high in glioma tissues, compared with adjacent normal tissues (P<0.05). Spearman's correlation analysis was then performed to analyze the association between miR-186 and IGF-1, which revealed that the expression of miR-186 was inversely correlated with that of IGF-1R in glioma (r=-0.7840; P=0.0003; Fig. 3C).

To investigate the direct interaction between miR-186 and its binding site in the 3'UTR of IGF-1R, a luciferase reporter assay was used. The pGL3-Wt-IGF-1R-3'UTR or pGL3-Mut-IGF-1R-3'UTR construct, with miR-186 mimics or miR-NC were transfected into HEK293T cells. The results indicated that the overexpression of miR-186 decreased the luciferase activity of pGL3-Wt-IGF-1R-3'UTR (P<0.05; Fig. 3D); however, the luciferase activity of pGL3-Mut-IGF-1R-3'UTR was not affected by simultaneous transfection with miR-186 mimics.

Western blot and RT-qPCR analyses were performed to evaluate the regulatory effects of the overexpression of miR-186 on the mRNA and protein expression of IGF-1R, respectively. The results showed that the overexpression of miR-186 significantly reduced the mRNA (P<0.05; Fig. 3E) and protein (P<0.05; Fig. 3F) expression levels of IGF-1R in the U251 and U87 cells. Collectively, these results demonstrated that miR-186 negatively regulated the expression of IGF-1R via binding to the 3'UTR of IGF-1R.

Knockdown of IGF-1R exerts similar effects as the overexpression of miR-186 in glioma. IGF-1R was identified as a direct target gene of miR-186. Therefore, it was hypothesized that IGF-1R contributes to the suppressive effects of miR-186 on glioma cells. To assess this, si-IGF-1R was transfected into U251 and U87 cells to knockdown the expression of IGF-1R. After 48 h, RT-qPCR and western blot analyses were performed to measure the mRNA and protein levels of IGF-1R. As shown in Fig. 4A and B, the expression levels of IGF-1R were reduced in the si-IGF-1R-transfected U251 and U87 cells (P<0.05). The results of the MTT and cell invasion assays revealed that the effects of si-IGF-1R on U251 and U87 cell proliferation (P<0.05; Fig. 4C) and invasion (P<0.05; Fig. 4D) were similar to those induced by the overexpression of miR-186. These results indicated that IGF-1R contributed to the tumor suppressive effects of miR-186 in glioma.
**Discussion**

Increasing studies have reported that the aberrant expression of miR-186 is a characteristic of malignancies. For example, miR-186 has been found to be downregulated in non-small cell lung cancer clinical tissues and cell lines, and the expression level of miR-186 was correlated with patient survival rates (18). Chen et al (19) demonstrated that the relative expression of miR-186 was lower in colon carcinoma tissues and cell lines. A study by Zhang et al (20) demonstrated that miR-186 was reduced in acute myeloid leukemia (AML). Patients with AML with low expression levels of miR-186 had significantly lower rates of complete remission and shorter overall survival rates, compared with patients with a high level of miR-186 (20). He et al (21) reported that miR-186 was commonly downregulated in esophageal squamous cell carcinoma (ESCC), and was correlated with the level of differentiation, tumor-node-metastasis stage and lymph node metastasis in patients with ESCC. Liu et al (22) found that miR-186 was expressed at low levels in multiple myeloma tissues and cell lines, and miR-186 has been shown to be downregulated in oral squamous cell carcinoma (23), bladder cancer (24), colorectal neuroendocrine tumors (25), gastric cancer (26) and hepatocellular carcinoma (27). These studies suggest that the downregulation of miR-186 is a frequent event in malignancies.
There is increasing evidence that miR-186 is important in tumorigenesis and tumor development. In non-small cell lung cancer, the upregulation of miR-186 targets CCND1, CDK2 and CDK6 to decrease proliferation by inducing G(1)-S checkpoint arrest (18). Cui et al (28) found that the enforced expression of miR-186 suppressed cell proliferation, migration and invasion through the downregulation of Rho-associated protein kinase 1 in non-small cell lung cancer. In colon carcinoma, the ectopic expression of miR-186 was found to significantly inhibit growth and metastasis via directly targeting Yin Yang 1 (19).

In ESCC, the re-expression of miR-186 was shown to suppress cell proliferation, invasion and enhance apoptosis through the negative regulation of S-phase kinase-associated protein 2 (21). Yao et al (24) demonstrated that the enforced expression of miR-186 reduces cell proliferation and invasion via the inhibition of nucleosome-binding protein 1 in bladder cancer. In multiple myeloma, the overexpression of miR-186 decreases cell growth in vitro and in vivo, and improves cell cycle G0/G1 arrest through the downregulation of Jagged 1 (22). A previous study also showed that the upregulation of miR-186 downregulates the mRNA and protein expression levels of YAP1, resulting in downregulation of the Hippo signaling pathway, and resulting in the repression of cell growth and metastasis in hepatocellular carcinoma (27). These findings implicate miR-186 as an attractive candidate therapeutic target in anti-tumor therapy.

In the present study, it was found that miR-186 was downregulated in glioma tissues, compared with that in adjacent...
normal tissues. The expression of miR-186 was also measured in glioma cell lines, and it was found that the expression levels of miR-186 were lower in glioma cell lines, compared with that in the normal HAC line. Subsequently, the functions of the overexpression of miR-186 in glioma cells were evaluated. The results of the MTT and cell invasion assays revealed that the upregulation of miR-186 suppressed the proliferation and invasion of glioma cells, respectively. This finding indicated that miR-186 may be a tumor-inhibiting factor.

To investigate the molecular mechanism underlying the involvement of miR-186 in the progression of glioma, the present study aimed to confirm the direct target genes of miR-186. According to bioinformatics analysis with several target prediction algorithms, IGF-1R was identified as a potential target gene of miR-186. A luciferase reporter assay was then performed to confirm this hypothesis. The results showed that miR-186 directly targeted the 3'UTR of IGF-1R. Subsequent experiments revealed that the mRNA expression of IGF-1R was high in glioma tissues and inversely correlated with the expression of miR-186. Subsequently, the induced expression of miR-186 decreased the mRNA and protein expression of IGF-1R in glioma cells. Finally, IGF-1R knockdown was found to exhibit similar tumor suppressive effects as that observed by miR-186 on glioma cell proliferation and invasion. These results indicated that miR-186 acted as a tumor-suppressive molecule in glioma genesis and progression, at least partially through regulating the expression of IGF-1R.

IGF-1R, a tyrosine kinase receptor, contains two extracellular α subunits with a ligand-binding site, two transmembrane β subunits and intracellular tyrosine kinase activity (29). It has previously been demonstrated that IGF-1R is involved in IGF-I and IGF-II signaling, and therefore regulates cell proliferation, cell cycle, apoptosis, metastasis, survival and differentiation (30-33). IGF-1R has been investigated widely in glioma. Harrington et al (34) found that IGF-1R was important in the tumorigenesis of glioma. In addition, studies have shown that apoptosis (35), growth (36-37), invasion (38), migration (39) and glucose metabolism (40) are regulated by IGF-1R in glioma. These findings suggest that selecting IGF-1R as a therapeutic approach is practicable for patients with glioma.

In conclusion, the present study showed that the miR-186/IGF-1R pathway regulated cell proliferation and invasion in glioma. These findings suggested that miR-186 may be a potential therapeutic target for the treatment of glioma in the future.

References