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MiR-93-5p inhibited proliferation and metastasis of glioma cells by targeting MMP2

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Abstract. – OBJECTIVE: To investigate the association between microRNA-93-5p (miR-93-5p) and glioma, and to explore the possible mechanisms.

PATIENTS AND METHODS: The expression level of miR-93-5p was detected in clinical tissue samples and cell lines. Online prediction websites were used to screen target of miR-93-5p, luciferase reporter assay and Western blot were performed to further confirm. The effects of the miR-93-5p on cell function were determined on U87-MG cells by *in vitro* experiments.

RESULTS: The low expression of miR-93-5p in glioma was confirmed by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay. In Target gene prediction, matrix metalloproteinase-2 (MMP2) was identified as a direct target of miR-93-5p. The subsequent experiments showed that decreased expression of MMP2 resulting from the up-regulation of miR-93-5p obstructed the cell proliferation ability of U87-MG cells, and G0/G1 block occurred during the growth cycle. Further, the invasion and migration ability were also been affected.

CONCLUSIONS: Our research emphasized the suppressive function of miR-93-5p in glioma by targeting MMP2, thus providing some novel experimental basis for the treatment of glioma.

Key Words:
MicroRNA-93-5p (miR-93-5p), Glioma, Matrix metalloproteinase-2 (MMP2).

Introduction

Glioma is the most common tumor of the central nervous system, accounting for about 40%-60% of intracranial tumors[1]. Currently, the clinical efficacy of surgical resection, chemotherapy, radiotherapy, and targeted therapy for glioma were not satisfactory. Worse still, the 5-year survival rate of less than 3-5% seriously threatened the health of glioma patients[2]. For this reason, more in-depth studies on glioma molecular biology and epigenetics are needed, so as to propose a new and effective treatment method. At present, glioma had been proved to had evolved from the multi-gene and multi-step development. Activation and overexpression of oncogenes lead to the release of malignant behavior of this kind of cells. Some studies[3] had found that micro ribonucleic acids (miRNAs) played a key role in the regulation of tumor gene expression and were closely related to the occurrence and development of malignant tumors.

MiRNA is a kind of non-coding single-stranded RNA with a length of about 18-25 nt, which has a high degree of evolutionary conservatism. It silences the target messenger RNA (mRNA) by binding to the three prime untranslated regions (3'UTR) of the target mRNA, thus inhibiting protein synthesis of the target gene and regulating gene expression[4]. More than 60% of human protein-coding genes contain miRNA binding sites in their 3'UTRs. At present, the miRBase database records more than 24,000 gene binding sites that encode more than 30,000 mature human miRNAs[5]. The number of target genes corresponding to a single miRNA could reach hundreds, and a certain number of miRNAs could also simultaneously regulate the same target gene. MiRNAs regulated cell phenotypic changes by regulating these target genes, resulting in changes in cell function[6].

MicroRNA-93-5p (miR-93-5p) belongs to the miR-106b-25 family, located on chromosome 11q22.1, and relatively conserved in mammals. In the recent literature on miRNAs and diseases, the relationship between miR-93-5p and cardiovascular disease was studied[7,8], and more research was showing strong regulation of miR-93-5p in cancer

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diseases. However, miR-93-5p played different roles in different cancers. As a cancer suppressor, the role of miR-93-5p in ovarian carcinoma[9] and breast carcinoma[10] was emphasized. On the other hand, miR-93-5p played an oncogenic role in non-small cell lung cancer (NSCLC)[11] and gastric cancer[12,13]. The regulation of miR-93-5p on cancer led us to associate it with glioma. Was miR-93-5p involved in the development of glioma? This assumption would be the focus of our experimental research.

Patients and Methods

Clinical Cases and Cell Lines

35 human glioma tissue specimens and brain tissues adjacent to the tumor through the enlarged resection were collected from patients who underwent the enlarged resection of glioma in the Neurosurgery Department in our hospital from December 2015 to December 2017. All patients did not receive chemotherapy, radiotherapy or other anti-tumor therapies before the surgery. This investigation was approved by the Ethics Committee of The Second Affiliated Hospital Zhejiang University School of Medicine. The signed written informed consents were obtained from all participants before the study.

The selected cell lines in this study were U87-MG highly malignant glioma cell line and LN-18 normal human brain glial cell line, which were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μmol/mL penicillin and streptomycin in an incubator with 5% CO_2 at 37°C. Then, the cells in good growth state were selected for subsequent experiments.

Transfection

According to the instructions of the new liposome Lipofectamine™ LTX and PlusReagent (2000) transfection reagents (Invitrogen, Carlsbad, CA, USA), transfection was carried out with Dulbecco's Modified Eagle Medium (DMEM) solution (Gibco, Rockville, MD, USA), and a blank control group was set up to transfect miR-93-5p mimics and LV-MMP2 at the same time. After 6 h, the culture solution was replaced with a DMEM solution containing 10% fetal bovine serum for further culture for 48 h.

The Target Gene Determination

For target gene prediction, the online prediction websites (TargetScan, miRDB, and microRNA) were used. And further target gene determination experiments, luciferase reporter assay, was essential. For luciferase reporter assay, in briefly, mutant-type and wild-type MMP-2 gene 3'UTR luciferase expression vectors (MUT-3'UTR and WT-3'UTR) were constructed and transfected with miR-93-5p mimics or miR-negative control (NC), respectively. Subsequently, they were jointly transfected into U87-MG cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the luciferase activity of each group of cells was detected using the luciferase reporter gene detector to identify whether MMP-2 was the direct target of miR-93-5p.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

The total RNA of each group of cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and operated according to the instructions of PCR kit (TaKaRa, Otsu, Shiga, Japan). PCR reaction conditions on ABI7300 real-time fluorescence quantitative machine: 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s for a total of 40 cycles. The relative expression level of mRNAs of each gene was expressed by the $2^{-\Delta\Delta Ct}$ value.

Western Blot (WB) Analysis

Radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China) was applied to lyse and extract the total cell protein, and bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) was used to measure the protein level. The loading buffer was added, protein denaturation was carried out at 100°C, and the loading amount per well was 20 μg. After Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed, the protein was transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by electricity. Then, the protein was blocked with 5% skim milk for 2 h, eluted with Tris-Buffered Saline with Tween 20 (TBST) for 3 times, incubated with the primary antibody (anti-MMP2, anti-β-actin) overnight. After that, the protein was eluted with TBST for 3 times, incubated with the secondary antibody for 2 h, followed by elution with TBST for 3 times. Finally, color development and fixation were carried out on X-ray films

in a dark room, and the protein semi-quantitative gray value was analyzed.

Cell Proliferation

U87-MG cells were inoculated in 96-well plates, and the cell proliferation ability after transfection was tested. From the time of transfection (0 h), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) was used to detect the proliferation of each group of cells at 24 h, 48 h, and 72 h respectively after transfection. The MTT assay was briefly summarized as follows: after culture, 20 mL of 5 mg/L MTT reagents were added to each well for incubation at 37°C for 4 h, and then, the culture medium was aspirated. 150 mL of dimethyl sulfoxide (DMSO) reagents (Sigma-Aldrich St. Louis, MO, USA) were added to each well, shaken on a shaker for 10 min under room temperature in a dark room, and tested on an enzyme reader to determine the absorbance value at the wavelength of 570 nm.

Cell Cycle Assay

Cells after transfection were digested with trypsin, collected *via* low-speed centrifugation, washed with the phosphate buffered saline (PBS) for three times and incubated with 70% ethanol solution at 4°C overnight. Then, cells were washed with PBS again and removed the supernatant and resuspended with about 100 μL of RNase, followed by incubation *via* water bath at 37°C for about 30 min. 100 μL of propidium iodide (PI) was added for incubation at 4°C for about 30 min in a dark place. The flow cytometry was used to detect the red fluorescence at an excitation wavelength of 488 nm, and the data were stored for analysis.

Transwell Assay

Cell invasion and migration assays were performed by transwell plates (Corning, Corning, NY, USA) with 8-μm-pore size membranes with Matrigel (for invasion assay) or without Matrigel (for migration assay). Briefly, 2×10^4 cells were planted into the upper chambers with serum-free medium. On the other hand, the lower chamber was offered with medium containing 10% fetal bovine serum as a chemoattractant. After 2 days incubating, the cells on the top of the membrane were wiped by a brush. Subsequently, the membrane was stained by 0.2% crystal violet followed drenched by 95% ethanol. The cells of migrating or invading were observed by an inverted microscope.

Statistical Analysis

Statistical analysis was performed with the Student's *t*-test or *F*-test. All *p*-values were two-sided and $p<0.05$ were considered statistically significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

MiR-93-5p Expression Found Reduced Both Tissues and Cells of Glioma

The presence of miRNAs with altered expression is the basis for studying the regulation of miRNAs. Based on the report of miR-93-5p in other related malignancies, we hypothesized that miR-93-5p also had similar regulatory effects in gliomas. Therefore, we first tested the expression level of miR-93-5p in clinical samples. In 35 samples of clinically diagnosed gliomas, miR-93-5p was ubiquitously expressed compared with normal brain tissues of the control group (Figure 1A). At the cellular level, we chose to detect miR-93-5p expression in the highly malignant glioma cell line (U87-MG) and normal human brain glial cell line (LN-18); surprisingly, compared with LN-18 cells, the expression of miR-93-5p in U87-MG cells also showed significant low expressed (Figure 1B). An apparent difference in miR-93-5p expression was found in glioma.

MMP2 is a Direct Target of MiR-93-5p in Glioma Cell

MiRNAs have multiple target genes, and the same target gene may also be regulated by different miRNAs. Therefore, the confirmation of target genes is particularly important to reveal the relevant mechanisms of miRNAs. The presence of online prediction websites facilitated miRNA research. In TargetScan, miRDB, and microRNA, MMP2 was found to have a pairing sequence with the miR-93-5p at the 3'UTR (Figure 2A). Then, we established luciferase reporter vectors containing the wild-type or mutant-type miR-93-5p seed sequences of the MMP2. The transfection efficiency of miR-93-5p mimics was confirmed firstly (Figure 2B). The results showed that upregulating miR-93-5p in U87-MG cells resulted in the decrease of the luciferase activity of the wide-type MMP2 reporter gene, but it had no effect on mutant-type (Figure 2C). To further validate the role of miR-93-5p in the negative regulation of MMP2, WB assay was performed, and the results were encouraging. Compared with the negative

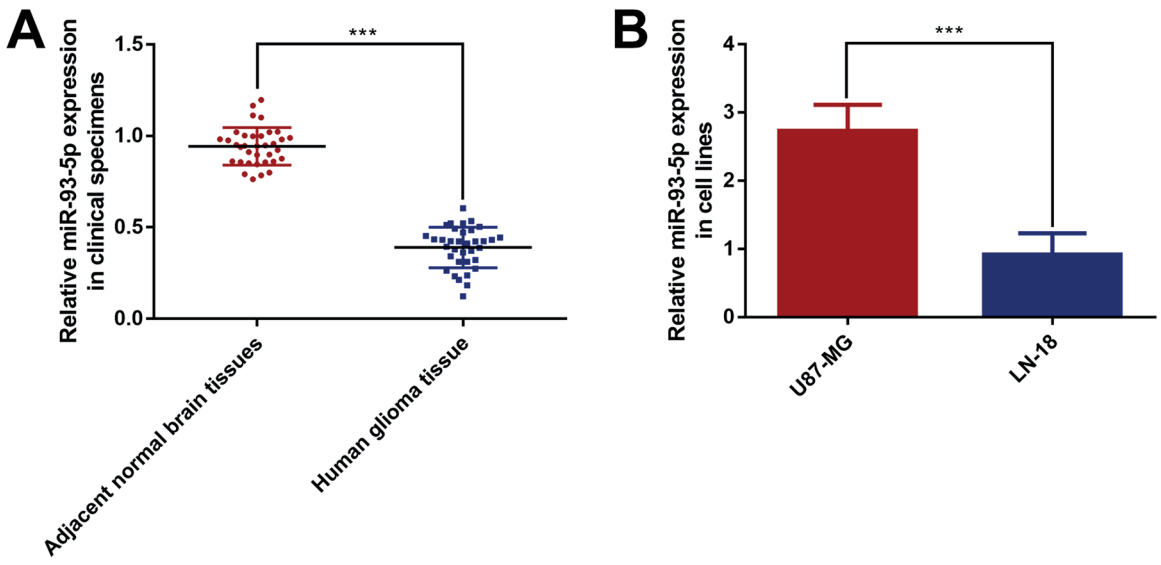

Figure 1. A, Difference in the expression of miR-93-5p between glioma tissues and corresponding adjacent normal brain tissues (***p<0.001). **B,** The expression of miR-93-5p in the malignant glioma cell line (U87-MG) and normal human brain glial cell line (LN-18) (***p<0.001).

control group, the expression of MMP2 protein in U87-MG cells transfected with miR-93-5p mimics was significantly decreased (Figure 2D). These series of experiments fully demonstrated the targeted regulation of miR-93-5p on MMP2 in gliomas.

MiR-93-5p Affected the Proliferation and Metastasis of Glioma Cells

The acquisition of abnormal proliferative capacity is an important marker for distinguishing between tumor cells and normal cells. In the cell function analysis, we found that miR-93-5p mimics could inhibit the proliferation of U87-MG cells. In the MMT experiment, there was a significant difference in cell proliferation ability from 48 h. The slope of the proliferation curve of U87-MG cells transfected with miR-93-5p mimics was significantly lower than that of the control group (Figure 3A). Further, we analyzed the changes of the cell cycle in different groups. In the miR-93-5p mimics group, U87-MG cells showed a distinct peak in the G0/G1 phase, which prompted the emergence of G0/G1 block. This seemed to be the reason why miR-93-5p inhibited the proliferation of U87-MG cells (Figure 3B).

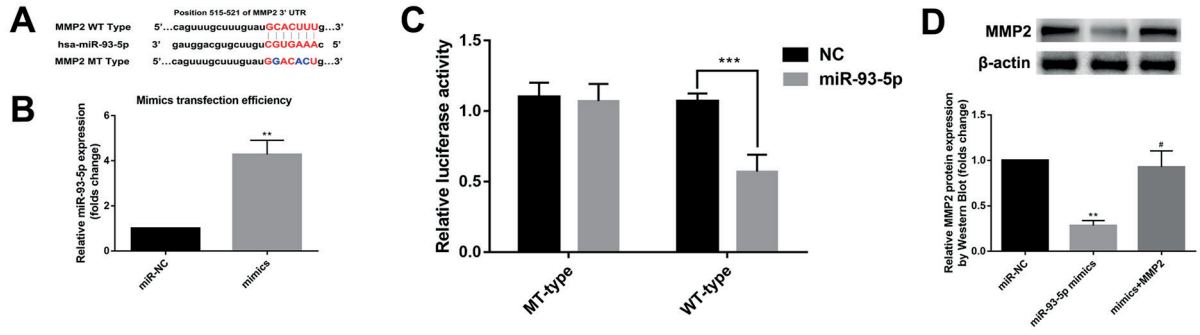

Figure 2. MMP2 is a direct and functional target of miR-93-5p. **A,** Diagram of putative miR-93-5p binding sites of MMP2. **B,** Transfection efficiency detection by qRT-PCR (**p<0.01). **C,** Relative activities of luciferase reporters (***p<0.01). **D,** The protein expressions of MMP2 in glioma cells after different treatment, Data were presented as means ± standard deviations (**p<0.01 *vs.* NC group; #p<0.05 *vs.* Mimics group).

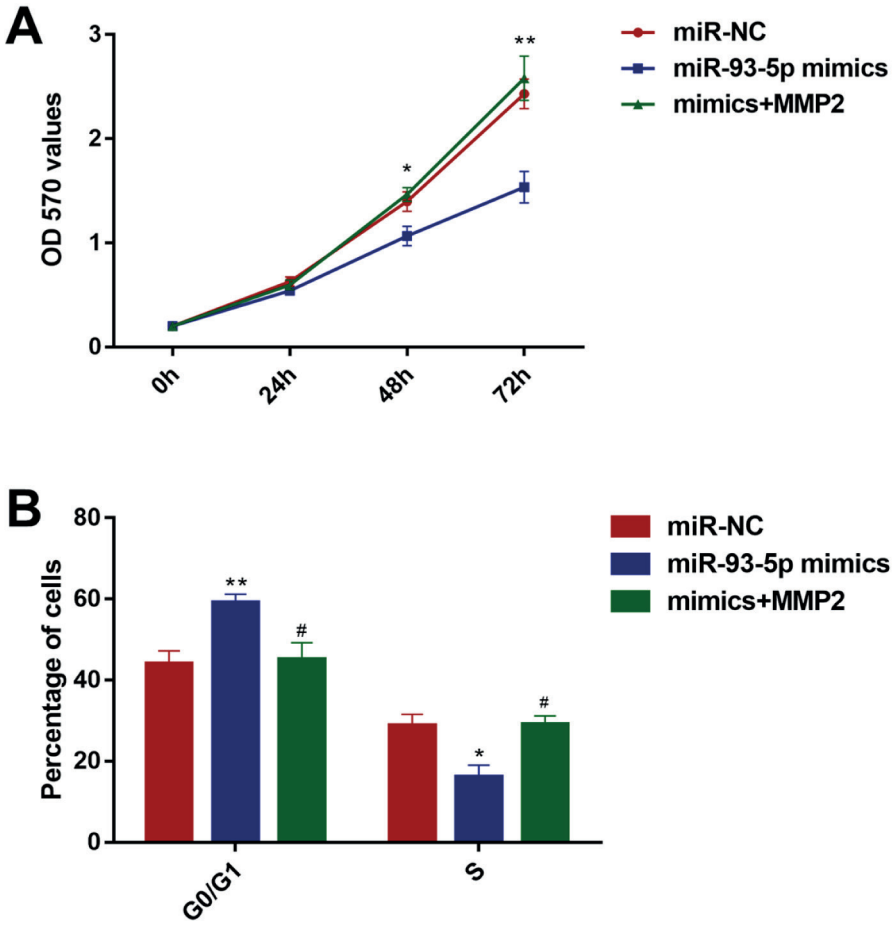

Figure 3. A, MiR-93-5p inhibited the proliferation of glioma cell (*p<0.05, **p<0.01 *vs.* NC group). **B,** The cell cycle phases of glioma cell analyzed using flow cytometry (*p<0.05, **p<0.01 *vs.* NC group; #p<0.05 *vs.* Mimics group). All data were presented as means ± standard deviations.

Invasion and migration were the essential manifestations of malignant tumors including gliomas; gliomas could show widespread invasion at an early stage. As a common experimental method for detecting cell invasion and migration, we used the transwell assay to analyze the metastasis ability of glioma cells. As shown in Figure 4, we found that miR-93-5p reduced the migration and invasion capacities of U87-MG cells.

In the process of cell function experiments, we have also set up a rescue experiment. We simultaneously transfected miR-93-5p mimics and LV-MMP2 into U87-MG cells to analyze whether the resume expression of MMP2 in U87-MG cells would interfere with the effects of miR-93-5p on cell function. Firstly, we observed in WB experiment that LV-MMP2 could increase the protein expression of MMP2 in U87-MG cells (Figure 2D). In subsequent functional experiments, we found that the proliferation and metastasis abilities of co-transfected U87-MG cells returned to the level of the control group (Figure 4).

Discussion

Glioma, originated from glial cells, is the most common malignant tumor of the central nervous system. According to CBTRUS statistics, glioma accounts for about 26.9% of all primary intracranial tumors and about 84% of all intracranial malignant tumors in the United States[14]. Glioma is characterized by high invasiveness, high recurrence rate, and high death rate. High invasiveness is the main cause of death of glioma, and due to its high invasiveness, glioma lacked a clear bound-

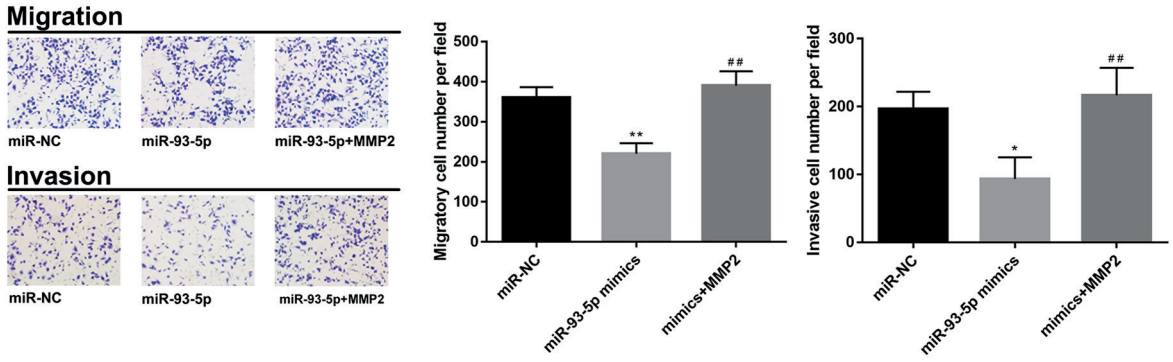

Figure 4. MiR-93-5p decreased the expression level of MMP2 and inhibits the invasion and migration of glioma cell. The invasion and migration were analyzed using transwell assay and detected by microscope (× 200). Data were presented as means ± standard deviations (*p<0.05, **p<0.01 *vs.* NC group; ##p<0.01 *vs.* Mimics group).

ary with normal brain tissues, so it was difficult to resect glioma completely[15]. Besides, glioma growing in important parts such as the brain stem, even cannot be surgically treated. At the same time, it was not very sensitive to radiotherapy and chemotherapy, very easy to relapse, and the survival rate of patients was pretty low.

At present, the comprehensive treatment of malignant brain tumors would no longer be the traditional surgery, radiotherapy, and chemotherapy. The rapidly developing molecular biology and genetic engineering technology provided a powerful weapon to solve this problem. In recent years, new progresses had been made in the involvement of miRNAs in the field of tumor research, which was mainly due to the action characteristics of miRNAs. According to the traditional concept, all kinds of proteins played a key role in regulating cell functions and signaling pathways in all organisms[16]. The translation of proteins must go through the processes of transcription from genes to specific mRNAs and then translation from mRNAs to amino acid chains[17,18]. MiRNAs could interfere in the process of gene translation into amino acid chains after transcription, thus affecting protein expression and further influencing its function. MiRNAs had a wide variety of types, with high quantification in cells and multiple targeting binding sites. These characteristics enable miRNAs to affect the expression of various important genes and their functions by targeting them and participate in the physiological and pathological processes of cells to play an important role[19,20]. In tumor-related aspects, miRNAs played an important regulatory role in the development of tumors[17]. More than 50% of human miRNA genes are located in tumor-related gene regions, including fragile regions, heterozygous loss regions, amplification regions or normal breaking point regions. These gene regions control important processes, such as cell proliferation, differentiation, and apoptosis[20]. These miRNA regulatory abnormalities exerted crucial effects on the occurrence, development, and metastasis of the tumor. Some miRNAs were poorly expressed in tumor tissues, and these miRNAs played a role in inhibiting oncogenes, thus preventing tumor occurrence. These miRNAs were called tumor suppressor genes. On the contrary, other miRNAs were highly expressed in tumor tissues and played a role in inhibiting tumor suppressor genes and promoting tumor development, as were called oncogenes[21]. In this study, the expressions of miR-93-5p in glioma tissue specimens and their paired adjacent normal brain tissues were compared, and it was found that miR-93-5p was significantly lowly expressed in samples of confirmed cases of glioma compared with that in normal brain tissues. The same result was also obtained at the cellular level. It was speculated that miR-93-5p played a role as suppressing oncogenes in the development of glioma.

The miRNA exerted its function by regulating the target gene. With the rapid development of bioinformatics software, the target gene of miRNAs was mainly determined by software prediction and cell experiment verification at present. In this work, it was also found that miR-93-5p was likely to target matrix metalloproteinase-2 (MMP-2) through the prediction of several bioinformatics software, which was verified by further cell experiments.

The ability of tumor cells to invade and migrate directly affected the spread of tumor cells, leading to increased difficulty in tumor treatment. Cell in-

vasion and migration were complicated pathological processes influenced by many factors, and the extracellular matrix (ECM) destruction was the key link of tumor cell invasion[22]. MMPs were a large family of extracellular proteolytic enzymes whose function was to degrade one or more protein components of the ECM[23]. MMP-2 was one of MMPs and also a major enzyme degrading the ECM, which had the ability to promote invasion and metastasis of malignant tumors[24]. When the tissue cells became malignant and form tumor cells, the tumor cells first fall off from the primary site, release MMP-2 after adhering to various components in the ECM through its surface receptors to degrade the framework in the ECM and destroy the protective barrier between cells, thus forming a local destruction zone. Then, they invaded the surrounding tissue or enter the blood vessel, ran under the influence of various factors and extravasated through the wall of the blood vessel to the secondary site to continue proliferation and formed a metastasis zone. Through a large number of studies, MMP-2 had been found to play an important role in many malignant tumors, such as gastric cancer[25], breast cancer[26], and ovarian cancer[27]. We found the binding target sequence of miR-93-5p and MMP2 on the target gene prediction site. Through further luciferase reporter and WB experiments, we confirmed the negative targeting regulation of miR-93-5p on MMP2.

Although studies had revealed that miR-93-5p was associated with the occurrence, development, invasion, and migration of multiple cancers, no studies have yet focused on its specific effect on glioma. In the functional *in vitro* experiments of our research, the biological effects of miR-93-5p on the proliferation and metastasis of U87-MG cells were studied; over-expressing miR-93-5p could effectively repress the proliferation and metastasis of U87-MG cells.

We showed that miR-93-5p/MMP2, as a target for the treatment of glioma, may become a feasible and new method of tumor treatment. However, more specific experimental mechanisms or research by *in vivo* experiments still need further exploration.

Conclusions

We showed the suppressive function of miR-93-5p in glioma by targeting MMP2, thus providing some novel experimental basis for the treatment of glioma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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