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MiR-5195-3p inhibits the proliferation of glioma cells by targeting BIRC2

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of microRNA-5195-3p (miR-5195-3p) on the proliferation of glioma cells and to explore its related mechanisms.

PATIENTS AND METHODS: The expression level of miR-5195-3p in glioma tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Online prediction websites (TargetScan and miRanda) were used to screen the potential targets of miR-5195-3p. Luciferase reporter gene assay and Western blot were performed to confirm the targets of miR-5195-3p. Furthermore, the effects of miR-5195-3p on cell proliferation were detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), colony formation assay, and flow cytometry, respectively.

RESULTS: MiR-5195-3p was lowly expressed in both glioma tissues and cells. Baculoviral IAP repeat-containing 2 (BIRC2) was identified as a direct target of miR-5195-3p. Over-expression of miR-5195-3p in glioma cells significantly decreased the protein expression of BIRC2. Besides, the proliferative capacity and colony formation ability were significantly inhibited after transfection of miR-5195-3p *in vitro*. In addition, flow cytometry indicated that an evident G1 phase arrest occurred in miR-5195-3p over-expressed group.

CONCLUSIONS: In this work, we emphasized the suppressor function of miR-5195-3p in the proliferation of glioma cells. Furthermore, our findings provided an experimental basis for the research and treatment of glioma.

Key Words:
MicroRNA-5195-3p (miR-5195-3p), Glioma, Baculoviral IAP repeat-containing 2 (BIRC2), Cell proliferation.

Introduction

Glioma is the most common type of malignant tumors in the central nervous system. It accounts for more than 70% of malignant brain tumors[1]. At present, comprehensive treatments including surgery, radiotherapy, and chemotherapy are widely used in clinic. However, the therapeutic effect is far from satisfactory. In recent years, new treatment methods, such as molecular targeting and immunosuppressive agents, have been adopted. However, the effect is still very limited. The average median survival time for gliomas patients is only about 14 months[2,3]. Besides, the recurrence rate of glioma is extremely high, with a 5-year survival rate less than 10%. This makes glioma one of the worst prognosis tumors.

MicroRNAs are a type of small, non-coding, endogenous single-stranded RNAs with about 19-25 nucleotides in length. The main biological function of microRNA is to regulate gene transcription, mainly by negative regulation[4,5]. In the process of tumorigenesis, incorrect DNA copy number, genome rearrangement, mutation, methylation, and other epigenetic mechanisms lead to changes in miRNA expression. This may further result in abnormal expression of tumor-related target genes, eventually leading to cancerization. In recent years, many studies have shown that abnormal expression of miRNAs is associated with the development of cancer and resistance to chemo-radiotherapy, including glioma[6-9].

MicroRNA-5195-3p (miR-5195-3p), a newly microRNA molecule, has not been fully studied. Recently, researches[10,11] have reported that miR-5195-3p inhibits the development of bladder cancer and non-small cell lung cancer, acting as a tumor suppressor gene. Based on the characteristics of invasive growth of glioma cells, the aim of this report was to investigate the effects of miR-5195-3p on the proliferation of glioma cells and to explore its related mechanisms.

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Patients and Methods

Clinical Cases and Cell Lines

30 human glioma tissue specimens were collected from patients undergoing surgical resection in the Neurosurgery Department of The First Affiliated Hospital of Henan University of Traditional Chinese Medicine from June 2016 to October 2018. Meanwhile, 10 brain normal tissues were obtained from normal subjects as control group. No patient received chemotherapy, radiotherapy or other anti-tumor therapies before the study. This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Henan University of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study.

Three highly malignant glioma cell lines (U251, U87, and U373) and one normal human brain glial cell line (LN-18) were used in this study. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 UI/μL penicillin and 100 μg/mL streptomycin in an incubator with 5% CO_2 at 37°C. Cells in good growth state were selected for subsequent experiments.

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

Total RNA in tissues and cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, extracted RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of the PrimeScript™ RT Master-Mix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 was used as an internal reference for miR-212-5p. Primer sequences used in this study were as follows: microRNA-5195-3p, F: 5'-GCCTGTAGGCATCATCGCCAG-3', R: 5'-GATAGAGTGACGTGAAGTAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Cell Transfection

MiR-5159-3p mimics, negative control miR-NC and LV-BIRC2 were transfected into cells according to the instructions of Lipofectamine 2000 liposome (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was confirmed by qRT-PCR. Three groups were established in this study, including miR-NC group (negative control), miR-5159-3p mimics group (U87 cells transfected with miR-5159-3p mimics), and mimics+BIRC2 group (U87 cell transfected with miR-5159-3p mimics and LV-BIRC2)

Target Gene Determination

Online prediction websites were first used to screen the potential targets of miR-5195-3p. BIRC2 was screened out to have a binding site with miR-5195-3p. Subsequently, the 3′-untranslated region (3′-UTR) of wild-type BIRC2 (Wt-BIRC2-3'-UTR, WT-type) and the 3'-UTR of mutant-type BIRC2 (Mut-BIRC2-3'-UTR, MT-type) were co-transfected with empty plasmid and miR-5195-3p overexpression plasmid into U87 cells. The cells were then cultured for 48 h, followed by dual-luciferase reporter gene assay. Next, the cells were washed with phosphate-buffered saline (PBS) 3 times. Finally, the luciferase activity of cells in each group was detected using the luciferase reporter gene detector.

Western Blotting

Cells with different treatment were first collected and lysed using radioimmunoprecipitation assay (RIPA) reagent (Yeasen, Shanghai, China). The supernatant was collected after centrifugation at 15000 rpm for 15 min. The concentration of extracted protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking in 5% skim milk powder for 1 h at room temperature, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL) chromogenic reagent was used for photographing. Target protein expression was finally calculated, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference.

Cell Proliferation

Transfected U87 cells of each group were digested and inoculated into 96-well culture plates. 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 cells 1-5 days after transfection. Briefly, 5 μL of MTT solution was added to each well at each time point, respectively. Meanwhile, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to dissolve blue-violet crystal after 4 h of incubation. After gently vibrating for 10 min, the absorbance of each well was measured by a microplate reader.

For colony formation assay, the cells were inoculated into 6-well culture plates (3 duplicates in each group) at a density of 1×10^3 cells per well. The culture plates were shaken slightly to achieve uniform distribution of cells. Subsequently, the cells were continuously cultured in an incubator until colonies could be observed by naked eyes. After discarding the culture medium, the cells were washed with phosphate-buffered saline (PBS) 3 times. Then, they were fixed with 2 mL of methyl alcohol for 15 min and stained with 2 mL of crystal violet dye for 15 min. After air drying, formed colonies were photographed, and the number of colonies in each group was counted.

For cell cycle: transfected cells were first digested and collected. After washing with PBS 3 times, the cells were incubated with 70% ethanol solution at 4°C overnight. Then, the cells were re-suspended in 100 μL RNase, followed by incubation in a water bath at 37°C for 30 min. 100 μL propidium iodide (PI) was added for incubation at 4°C for 30 min in dark. Flow cytometry was used to detect the red fluorescence at an excitation wavelength of 488 nm.

Statistical Analysis

Prism 7.02 software (La Jolla, CA, USA) was used for all statistical analysis. Student's *t*-test was used to compare the difference between the two groups. All *p*-values were two-sided, and $p<0.05$ was considered statistically significant.

Results

MiR-5195-3p was Lowly Expressed in Glioma Tissues and Cells

Abnormal changes in the miRNA expression profile are involved in the occurrence and development of many diseases[12-14]. In our research, we first detected the expression level of miR-5195-3p in clinical glioma tissues. Among the 40 clinical samples (including 30 glioma samples and 10 normal samples), we found that the expression of miR-5195-3p in glioma tissues was significantly lower than that of normal brain tissues (Figure 1A). Subsequently, we detected miR-5195-3p expression in three malignant glioma cell lines and one normal glial cell line as well. Similarly, the expression of miR-5195-3p in malignant glioma cell lines was obviously down-regulated. The expression level of miR-5195-3p in U87 cells was the lowest (Figure 1B). Therefore, U87 cells were chosen for further experiments.

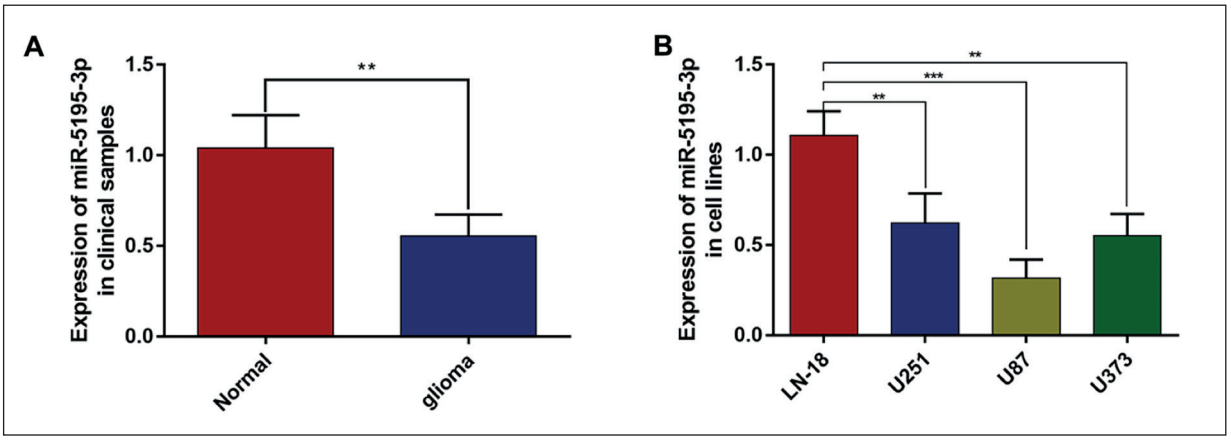

Figure 1. Expression of miR-5195-3p in glioma tissues and cells. **A,** Expression of miR-5195-3p in tissues (**$p<0.01$). **B,** Expression of miR-5195-3p in cells. (**$p<0.01$, ***$p<0.001$).

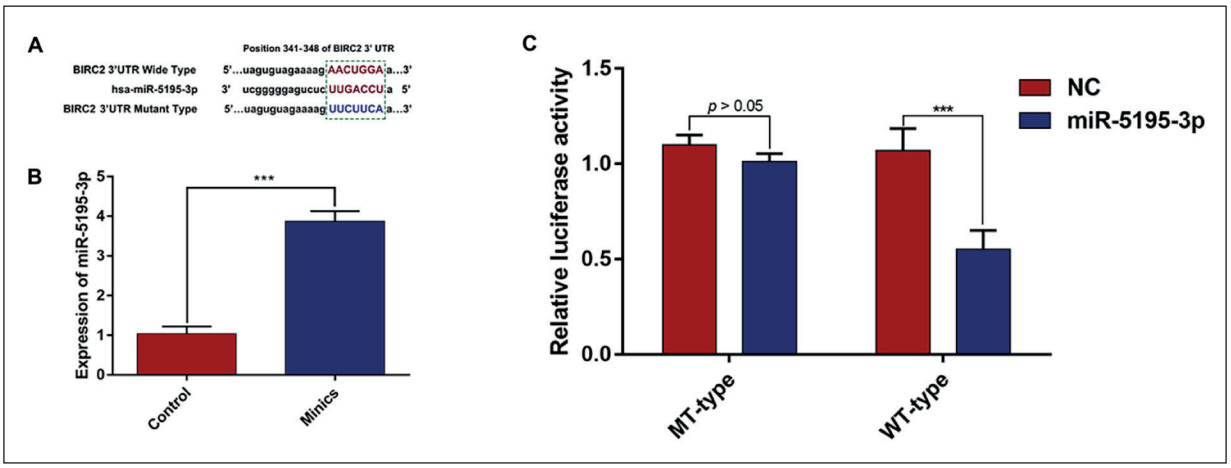

Figure 2. BIRC2 was a direct and functional target of miR-5195-3p. **A,** Diagram of putative miR-5195-3p binding sites of BIRC2. **B,** Transfection efficiency detected by qRT-PCR. (***p<0.001). **C,** Relative activities of luciferase reporters. (***p<0.001).

BIRC2 was a Direct Target of MiR-5195-3p in Glioma Cells

The identification of target genes is particularly important to reveal the relevant mechanisms of miRNAs[15]. Combining the most commonly used online forecasting websites, BIRC2 was identified as a downstream target of miR-5195-3p (Figure 2A).

To verify the regulatory effect of miR-5195-3p on BIRC2, miR-5195-3p mimics were transfected into U87 cells. QRT-PCR results indicated that miR-5195-3p mimics could significantly increase the expression of miR-5195-3p in U87 cells (Figure 2B). Subsequent luciferase reporter gene assay demonstrated that up-regulating miR-5195-3p resulted in significantly decreased luciferase activity of wide-type BIRC2 reporter gene. However, it had no effect on the mutant-type BIRC2 reporter gene (Figure 2C). These results revealed the direct regulation of BIRC2 by miR-5195-3p.

MiR-5195-3p Decreased the Protein Expression of BIRC2

MiRNAs can affect the expression of target genes through post-transcriptional level[5]. In this study, Western blotting illustrated that the protein expression level of BIRC2 in glioma cells transfected with miR-5195-3p mimics was significantly suppressed. This once again demonstrated the targeted regulation of miR-5195-3p on BIRC2. On the other hand, we found that LV-BIRC2 could markedly enhance the protein expression of BIRC2 in transfected glioma cells (Figure 3).

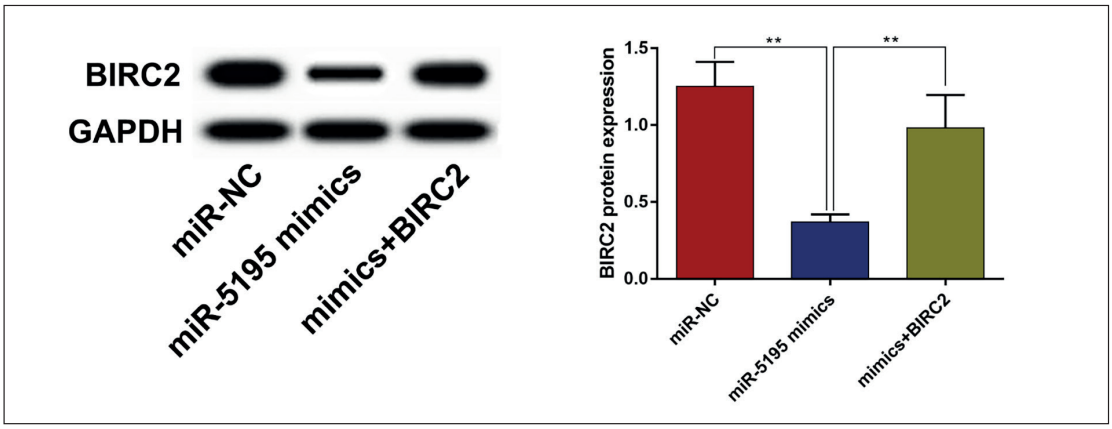

Figure 3. The protein expression of BIRC2 in glioma cells after different treatment. Data were presented as means ± standard deviations. (**p<0.01).

MiR-5195-3p Affected the Proliferation of Glioma Cells

MTT assay showed that the OD value of U87 cells in miR-5195-3p overexpression group was significantly lower than the other two groups. Meanwhile, the proliferation curve was also remarkably inhibited (Figure 4A). This indicated that miR-5195-3p affected the growth of glioma cells. Colonies formation assay indicated that, compared with the other two groups, the number of formed colonies in miR-5195-3p mimics group was significantly less and the size was smaller (Figure 4B). Furthermore, we analyzed the changes of the cell cycle in different groups. The results showed that the proportion of cells in G1 phase increased significantly in miR-5195-3p mimics group (Figure 4C). However, LV-BIRC2 transfection could significantly reverse the effects of miR-5195-3p in the above three experiments.

Discussion

In the past 20 years, the median survival time of glioma patients has not improved significantly. It is difficult to achieve radical cure in various clinical therapies. Meanwhile, recurrence occurs in almost all patients with glioma[16]. Therefore, an in-depth study on the proliferation characteristics of glioma and the search for effective therapeutic targets have become the key for glioma treatment.

Since the discovery of miRNAs for the first time in 1993[17], more and more miRNAs have been found. MiRNAs have been confirmed to play important roles in cell proliferation, differentiation, apoptosis[18-20]. Meanwhile, they participate in the occurrence and development of various diseases, including malignancies[21]. Currently, researches have found that miRNA can be used as a potential therapeutic target for glioma patients. For example, miR-25 plays an oncogenic role in human glioma by targeting CDKN1C. Moreover, it can potentially be a therapeutic target for glioma intervention[22]. MiR-590-3p has been found to inhibit the migration, invasion, and EMT of glioma cells, thereby affecting malignant glioma progression[23]. MiR-136 plays a key role in TMZ resistance by targeting AEG-1 in glioma. This suggests that miR-136 can be used to predict the response of patients to TMZ therapy[24].

To explore the specific function of miR-5195-3p in glioma, qRT-PCR was first conducted to detect the expression of miR-5195-3p in glioma tissues and cell lines. The results showed that the expression of miR-5195-3p was significantly down-regulated in both glioma tissues and cells. These data suggested that miR-5195-3p might act as a tumor suppressor gene in glioma, similar to its role in other malignant tumors[10,11].

MiRNAs may affect the activity of cancer cells, the function of which is related to target genes[25]. Therefore, it is necessary to further identify and verify the downstream target genes of miRNAs. In this study, prediction websites of TargetScan and miRanda were used to bioinformatically predict the target genes of miR-5195-3p. BIRC2 was finally screened out as a potential target gene for miR-5195-3p in glioma. Dual

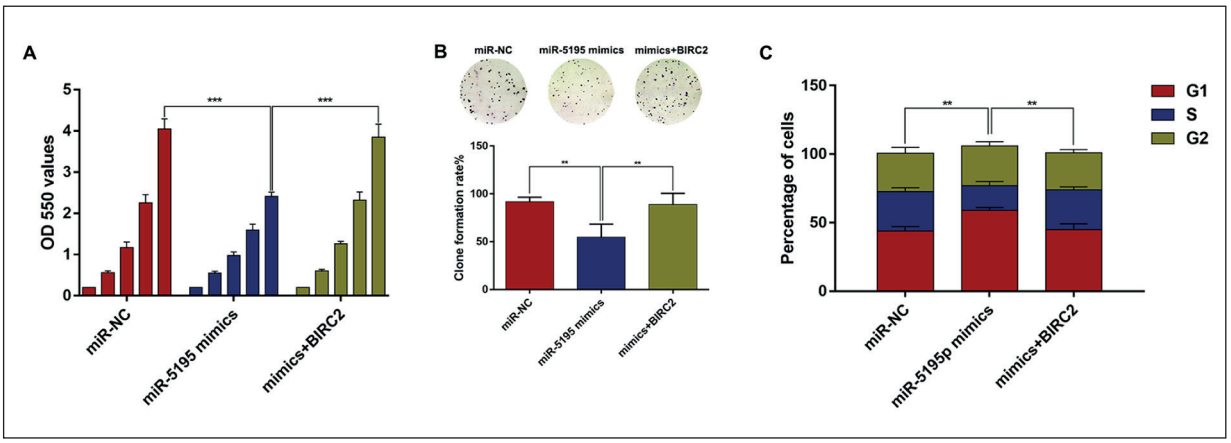

Figure 4. MiR-5195-3p inhibited the proliferation of glioma cells. **A,** Cell proliferation detected by MTT assay. (***p<0.001). **B,** Clone formation detected by colony formation assay (magnification × 40). (**p<0.01). **C,** Cell cycle phases of glioma cells analyzed using flow cytometry. (**p<0.01). Data were presented as means ± standard deviations.

luciferase reporter gene assay indicated that the luciferase activity of reporter vector containing WT-type was significantly lower than the reporter vector with Mut-type. Meanwhile, we found that the protein level of BIRC2 was significantly suppressed in miR-5193-3p over-expressed cells. Taken all, these results suggested that BIRC2 was the direct target gene of miR-5195-3p in glioma.

BIRC2 is a member of the anti-apoptotic gene family. Its proteins include three motifs: Baculoviral Inhibitor of Apoptosis Repeat (BIR), Really Interesting New Gene (RING), Caspase Recruitment Domain (CARD)[26]. The sequence of BIRC2 is highly conservative, which inhibits apoptosis by interfering with the activation of caspases[27]. Yamato et al[28] have shown that BIRC2/3-NIK signaling target effectors other than NF-kB contribute directly to carcinogenesis. Furthermore, the carcinogenicity of BIRC2 has been demonstrated in a variety of tumors[29,30]. Our *in vitro* experiments demonstrated that miR-5195-3p overexpression significantly inhibited cell proliferation and induced G1 arrest. In addition, colony formation assay showed that miR-5195-3p overexpression significantly inhibited the proliferation of glioma cells.

Conclusions

MiR-5195-3p significantly inhibited the proliferation of glioma cells by targeting BIRC2. Furthermore, miR-5195-3p/BIRC2 might be a potential target for glioma therapy. However, more specific experimental mechanisms or research still need exploration.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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