Original Article

miR-199 modulates Wnt/β-catenin signaling pathway to involve in the progression of brain stem glioma

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Abstract: Objective: To explore the mechanism of miR-199 involving in targeted modulation of Wnt/β-catenin signaling pathway in glioma cells and the potential value of miR-199 acting as a therapeutic target as well as a prognostic sign of malignant glioma, by analyzing the expression difference of miR-199 in different levels of brain stem glioma. Methods: microRNA expression profile obtained from previous researches on different levels of brain stem glioma was used to analyze and screen differentially expressed microRNA. 28 cases of brainstem glioma specimen and 8 cases of normal brain tissue specimen that collected from March 2013 to March 2015 in the Department of Neurosurgery of our hospital were selected in this study to detect the expression of Wnt-2, mir-199a-5p and β-catenin in different levels of brainstem glioma tissues and normal brain tissues by using qRT-PCR, Western Blot and immunohistochemistry method. We also analyzed the correlation between miR-199a-5p and Wnt-2 as well as the relationship of clinical pathology between miR-199a-5p and brainstem glioma. The results of luciferase assay proved that miR-199a-5p target-modulated Wnt2. We infected U251 cells with miR-199a-5p over-expressed lentivirus, and CCK-8, flow cytometry and Transwell assay verified that MIR-199a-5p involved in the modulation of proliferation, apoptosis and invasiveness of U251 cells through target inhibition of Wnt/β-catenin signaling pathway, and confirmed the role of miR-199/Wnt2/β-catenin signaling axis in the progression of brain stem glioma. Results: qRT-PCR detection showed that the expression of miR-199a-5p in normal brain tissues and low-grade brainstem glioma tissues was significantly higher than that of the high-grade brainstem glioma tissues (P < 0.01, P < 0.05). Western Blot assay showed that the expression of Wnt2 and β-catenin in normal brain tissues and low-grade brainstem glioma tissues was significantly lower than that in the high-grade brainstem glioma tissues (P < 0.01, P < 0.01); immunohistochemistry results showed that Wnt2 expressed in Cytoplasm and membrane, and the expression of Wnt2 in tissues of normal brainstem and low-grade brainstem glioma was significantly lower than that in the high-grade brainstem glioma tissues (P < 0.01, P < 0.01). Luciferase assay showed that miR-199a-5p mimic significantly inhibited the activity of Renilla luciferase in 293 cells (P < 0.05), which confirmed that miR-199a-5p target-inhibited the expression of Wnt2. The CCK-8 growth curve showed that proliferation of U251 cells was significantly inhibited after the over-expression of miR-199a-5p (P < 0.01). Flow cytometry showed that miR-199a-5p promoted the apoptosis of U251 cells. Transwell assay confirmed that miR-199a-5p had significantly inhibited the invasion of U251 cells. Conclusion: Decreased expression of miR-199a-5p in brainstem glioma resulted in abnormal activation of Wnt2/β-catenin signaling pathway, and the expression level of miR-199a-5p in brainstem gliomas was negatively correlated with the degree of tumor malignancy. For brainstem gliomas, miR-199a-5p can be used as a prognostic target and is promising to become a potential target for the treatment of tumors.

Keywords: miR-199, Wnt2/β-catenin, brain stem gliomas

Introduction

Technologies in chemotherapy, radiotherapy and microneurosurgery have been constantly improved nowadays, but the overall prognosis of patients with brainstem glioma is still not satisfactory. The 2-year survival rate is no more than 25%, the median survival time is about 9 to 12 months, and the median progression free survival time is about 5 to 6 months, the prognosis is not ideal [1-4].

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Study on the Wnt/β-catenin signaling pathway

during its evolution Wnt/β-catenin signaling pathway plays an important role in the occurrence and progression of many kinds of tumors, including glioma [5]. The study showed that there is an abnormal activation of Wnt/β-catenin signaling pathway in brain glioma, however, the mode of Wnt/β-catenin pathway activation in glioma is not very clear [6].

MicroRNA is a type of endogenous non-coding single-stranded RNA that found in eukaryotic cells and viruses; it down-regulates the expression of genes in the cells by inhibiting the translation of mRNA or promoting the degradation of mRNA. The studies on microRNA and tumor show that microRNA participates in a variety of physiological processes through the regulation of gene expression in tumor cells [7].

In the early stage of this research, by using microRNA expression profile chip, we found that multiple microRNAs expressed differently in the tissues of brain stem glioma at different levels. By using bioinformatics analysis, we found miR-199a-5p in differently expressed microRNAs may target regulate Wnt2 expression. Therefore, on the basis of the previous works, we planned to study the potential mode of miR-199a-5p regulating Wnt/β-catenin pathway and its expression in glioma as well as its relationship with the degree of malignancy and prognosis by using molecular biology and cell biology methods, to provide more theoretical basis for the targeted treatment of brain stem glioma in the future.

Material and methods

Main reagents and instruments

Mouse anti-human GAPDH monoclonal antibody (Santa cruz, America), Mouse anti-human β-catenin monoclonal antibody (Millipore, America), Sheep anti-human Wnt-2 polyclonal antibody (Santa cruz, America), Sheep anti-human c-MYC polyclonal antibody (Santa cruz, America), Sheep anti-human cyclinD1 polyclonal antibody (Santa cruz, America), Sheep anti-human BAX polyclonal antibody (Santa cruz, America), Mouse anti-human Bad polyclonal antibody (Santa cruz, America), Mouse anti-human caspase3 polyclonal antibody (CST, America), Sheep anti-human MMP2 polyclonal antibody (Santa cruz, America), Sheep anti-human MMP9 polyclonal antibody (Santa cruz, America), QRT-PCR microRNA test kit (Guangzhou Gene Copeia Co., Ltd.), FITC/PI double staining apoptosis detection kit (Tianjin Sun gene Biotech Co., Ltd.), miR-199a-5p mimic (Shanghai Gene Pharma Co., Ltd.), MIR-199a-5p over expressed lentivirus and control lentivirus (Shanghai Rui Sai Biotechnology Co., Ltd.), Wnt2-3'UTR luciferase reporter plasmid (Shanghai Rui Sai Biotechnology Co., Ltd.), U251 cell (ATCC), 293 cell (ATCC), DMEM medium (GIBCO, America), fetal bovine serum (GIBCO, America), CCK8 Kit (Dojindo, Japan), Traswell chamber (Corning, America), automatic photographic device and fluorescence inverted microscope (Nikon, Japan), flow cytometer (BD FASAria cell sorter), frozen tissue slicer (Beijing Huaxing instruments Science & Technology Development Co., Ltd.), immunohistochemistry staining kit SP-9000 (Beijing Zhong Shan Jinqiao Biological Technology Co., Ltd.) and ECL Kit (Shanghai Jingke Chemical Technology Co., Ltd.).

Tissue samples

Tissue samples were selected from the patients admitted in the Department of Neurosurgery of our hospital from March 2013 to March 2015. Inclusion criteria: (1) The estimated survival time was more than 3 months; (2) Kamofsky score was more than 60 points; (3) No previous surgery, radiotherapy or chemotherapy before inclusion; (4) It was diagnosed as brainstem glioma by MRI. Exclusion criteria: (1) Severe liver and kidney dysfunction; (2) Abnormal blood cell counts; (3) Combined infection; (4) Pregnant or breast-feeding women, etc. All patients or their families were informed and signed informed consent. First, the tissues were removed within 30 min and then stored in liquid nitrogen for use. According to the central nervous system tumor classification enacted by World Health Organization in 2007, the brainstem gliomas were divided into low-grade gliomas and high-grade gliomas. 28 cases of brainstem glioma in this study included 10 cases of high-grade and 18 cases of low-grade, as comparison, there were 8 normal samples taken from the surrounding brain tissues of primary epilepsy patients. The collection of the human brain tissues was in compliance with the provisions of the ethics committee of our
Study on the Wnt/β-catenin signaling pathway

hospital, and the agreement was obtained from patients or their families.

qRT-PCR

50 mg brain tissue were completely smashed by homogenate machine (operate on ice), add 1 ml Trizol into the homogenate for cell lysis, then transfer the lysate into a centrifuge tube, and repeated beat upon the lysate with a 1 mL pipette tip until there was no visible precipitation and place at room temperature for 5 min for complete lysis of nucleic acid protein. Add 250 µl chloroform into each tube, hand reverse EP tube upside and down for 15 s, keep it at room temperature for 15 min, and then centrifuge at 12000 RPM under 4°C for 15 min. Move the supernatant to a new 1.5 ml EP tube, then add the same volume of isopropanol, which was pre-cooled at the temperature of -20°C, and precipitate the mixture for 10 min at the temperature of -20°C. Then centrifuge at 12000 rpm for 10 min at 4°C, discard the supernatant, add 1 ml 75% ethanol that pre-cooled at 4°C, wash the precipitation, and then again centrifuge at 10000 rpm for 5 min under 4°C, discard the supernatant and dry the precipitation under room temperature. Completely dissolved RNA in 20 µl RNase-free water, and then analyze the concentration of RNA by UV. Then qRT-PCR was performed according to the operating instructions of the microRNA detection kit, and U6 snRNA was used as a reference gene for relative quantitative analysis.

Western blot detection

Take 100 mg brain tissues and grind under liquid nitrogen condition, then add 5080 µl lysate to break down protein for 30 min on ice, then centrifuge at 12000 r/min for 20 min under 4°C, take supernatant and detect the protein concentration by Bradford assay. Prepare 5% stacking gel and 10% separation gel, and then 80 g protein loading was used for PVDF membrane semi-dry trans-blot with vertical SDS-PAGE electrophoresis, and later use 5% defatted milk powder to block for 60 min. Wnt-2, β-catenin primary antibodies were diluted at 1:500, 1:500 respectively, and incubated in room temperature for 3 h and then at 4°C overnight, HRP labeled IgG secondary antibody (1:2000) and mouse anti-human GAPDH monoclonal antibody (1:5000) were incubated for 1 h under room temperature, and then add the detection reagent, expose, and carry on the grey analysis of the strips by using Quantity One 4.22, relative value of target protein expression = gray value of target belt/gray value of b-actin belt in the same sample.

Immunohistochemistry testing

The tissue specimen from 28 cases of brainstem glioma and 8 cases of normal brain were used to prepare 6 μm frozen slices at -20°C. After air-dried, fixed the slices with acetone for 10 minutes at 4°C, then incubated in 3% GU H2O2methanol solution for 10 minutes before blocked with normal serum for 30 minutes, Sheepanti-Human Wnt-2 polyclone antibody (dilution ratio: 1:20), Rabbitanti-Human Wnt-1 polyclone antibody (dilution ratio: 1:50), Mouseanti-Human C-myc, β-catenin and Ki-67 antibodies (dilution ratio: 1:50, 1:60, 1:100 respectively), overnight at 4°C, secondary antibody was incubated for 20 minutes at room temperature, then used DAB solution to show color, then used hematoxylin to counterstain before mounted. PBS was used as negative control. Double blind method was used to calculate test results, if brown staining appeared in nucleus or cytoplasm, the immunohistochemical result of β-catenin was positive; if brown stain appeared in cell membrane or cytoplasm a proximal to cell membrane, the results of Wnt-1 and Wnt-2 were positive; if dark brown or brown appeared in cell nucleus, then c-myc was positive. Under the microscope ×200, selected 5 to 10 fields in the strongest immune region of each slice with cell count no less than 500 to score the immunoreactivity according to the positive cells percentage and carry on semi quantitative analysis. The score standards of positive cells percentage: 0 for positive cell percentage < 1%; 1 point for 1%~10%; 2 points for 10%~25%; 3 points for 25%~50%; and 4 points for > 50%. The percentage of positive cells in 1000 cells was defined as the Ki-67 labeling index.

Cell culture

U251 cells and 293 cells were inoculated into 10 cm culture dishes separately and cultured with culture medium (DMEM + 10% FBS + 1% P/S + 1% Glutamax) under the condition of 5% CO2 and 37°C.
Study on the Wnt/β-catenin signaling pathway

Figure 1. Detection of miR-199a-5p and Wnt2/β-catenin in tissue samples. A. Expressions of miR-199-5p in tissues of brainstem glioma at different levels and normal brain examined by qRT-PCR. B. Expressions of Wnt2 and β-catenin in tissues of brainstem glioma at different levels and normal brain (×200) examined by immunohistochemistry. C. Expressions of Wnt2 and β-catenin in tissues of brainstem glioma at different levels and normal brain examined by Western Blot.

Cell transfection and lentivirus infection

10 pmol miR-199a-5p mimics and 0.5 µl POLO3000 transfection reagent were used to prepare the transfection mixture. The 293 cells were inoculated into 24 orifice plate (10⁵/holes) to culture overnight; then removed the culture medium and replenished fresh culture medium (500 µl DMEM + 10% FBS, without antibiotics), uniformly add transfection mixture into cells in the 24 orifice plate, gently shaked the cell culture plate and then wrapped in tinfoil to incubate at 37°C.

The U251 cells were inoculated into 6 orifice plate (5×10⁵/holes) to culture overnight under the conditions of 5% CO₂ and 37°C. On the second day, suitable amount of miR-199a-5p over-expressed lentivirus (miR-199-OE) and negative control lentivirus (NC) liquid (MOI = 30) were added into the culture respectively, in the meanwhile, 8 µg/ml polybrene was added in to enhance infection.

MicroRNA targeted analysis

TargetScan 7.0, miRBase, starBase 2.0 and other analysis softwares and databases were used to predicatively analyze the target genes of miR-199-5p. For those predicted potential target genes of miR-199-5p, GO function clustering and KEGG pathway analysis were used to screen the best to-be-tested target genes and related signaling pathways.

Luciferase assay

10 pmol miR-199a-5p mimics, 200 ng Wnt2-3'UTR Reporter plasmid (wild-type or miR-
199a-5p binding site deletion mutant type) and 0.5 µl POLO3000 transfection reagent were used to prepare the transfection mixture. The 293 cells were inoculated into 24 orifice plate (10^5/hole); removed culture medium when cell fusion reached 50%, and replace with 500 ul fresh culture medium (DMEM + 10% FBS, without antibiotics), uniformly added transfection mixture into cells in the 24 orifice plate. Gently shake culture plate and then wrap in tinfoil to incubate at 37°C. After 48 hours, collect cells and detect the luciferase activity according to the instruction of Dual-Glo luciferase assay system.

**Growth curve drawing**

The U251 cells of miR-199-OE group and NC group were inoculated into 96 orifice plate (10^4/hole), and CCK8 was used to detect the cell viability from day 1 to day 6. The detection methods: 10 ul CCK-8 was added into each hole and incubated in the incubator for 2 h after mixing it up, and then the light absorbance value at 450 nm were examined. The OD value sat 450 nm in miR-199-OE group and NC group were examined by microplate reader recorded as measured value and blank value, respectively. Final value = Measured value - Blank value.

**Flow-cytometry to examine cell apoptosis**

Trypsin was used to digest adherent U251 cells of miR-199-OE group and NC group; the cells were centrifuged and collected, and then washed by PBS twice. Again, the cells were centrifuged at 1000 rpm for 5 min and then collected. 400 µl binding buffer was used to re-suspend cells of each sample. FITC/PI double staining reagent was added and gently mixed, and then incubated for 30 min at 20°C without light. Cell apoptosis was detected by flow-cytometry.

**Transwell assay**

Before inoculating U251 cells of miR-199-OE group and NC group, add 30 µl diluted matrigel to Transwell inserts, stand by for 3 h at 37°C; cells were digested with pancreatin and then counted, adjust cell concentration to 4×10^5/ml; 0.1 ml cell suspension was added into Transwell inserts, at the same time, 0.6 ml complete

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**Figure 2.** MiR-199a-5p target-inhibited Wnt2 expression in glioma cells. A. Binding sites of miR-199a-5p analyzed and predicted by bioinformatics. B. Relative activity of luciferase in U251 cells after co-transfection examined by luciferase assay. C. Expression of Wnt2 in U251 cells after transfection examined by Western Blot. ***P < 0.001 vs. scramble group.
medium was added in the lower orifice plate; Transwell inserts were carefully placed into orifice plate filled with culture medium by tweezers (avoid making bubble at the bottom) and incubated in a incubator; observe the migration every day and stop incubation at 30 h; take out Transwell inserts, remove the supernatant, and carefully wipe the cells on upper level of the inserts with wet cotton swab, and then wash with PBS; 0.5 ml 4% paraformaldehyde was added into a clean hole, and Transwell insert was put back into the hole at room temperature for 15 min. Then use crystal violet to dye cells and evaluate the cell invasion by testing the OD value (570 nm).

**Statistical methods**

SPSS17.0 was used to analyze the data, and one-factor analysis of variance was used to compare the expression levels of microRNA and protein in the tissues of normal brain and brainstem glioma. The difference between groups was tested by LSD-t; a $P < 0.05$ was regarded as statistically significant.

**Figure 3.** The activity of Wnt2/β-catenin signaling pathway in U251 cells was inhibited after MiR-199a-5p over-expressed lentiviral infections. A. Picture of U251 detected by GFP fluorescence assay after lentivirus infections (left, 200 times) and expression of miR-199a-5p after lentivirus infections (right). B. Expression of Wnt2 and β-catenin in U251 cells after lentivirus infections examined by Western Blot assay.
Study on the Wnt/β-catenin signaling pathway

Results

Detection of miR-199a-5p and Wnt2/β-catenin in tissue samples

qRT-PCR detection showed that the expressions of miR-199a-5p in normal brain tissues and low-grade brainstem glioma tissues were significantly higher than that in high-grade brainstem glioma tissues (Figure 1A, P < 0.01, P < 0.01); immunohistochemical results showed that Wnt2 expressed in the cytoplasm and cytomembrane, and the expressions of Wnt2 and β-catenin in normal brain tissues and low-grade brainstem glioma tissues were significantly lower than that in high-grade brainstem glioma tissues (Figure 1B, P < 0.01, P < 0.01); Western Blot results also showed that the expression of Wnt2 and β-catenin in normal brain tissues and low-grade brainstem glioma tissues were significantly lower than that of high-grade brainstem glioma tissues (Figure 1C, P < 0.01, P < 0.05).

MiR-199a-5p target inhibited Wnt2 expression

Bioinformatics analysis showed that there was a predicted binding site of miR-199a-5p at nucleotide segments in 3'UTR region of Wnt2 gene (Figure 2A). Luciferase assay showed, comparing with mimic NC (scramble) group, the relative activity of renilla luciferase was significantly inhibited (P < 0.01) in 293 cells after co-transfected with miR-199a-5p mimic and wild-type Wnt2 3'UTR luciferase Plasmids, while the relative activity was not significantly affected after co-transfected with miR-199a-5p mimic and mutant Wnt2 3'UTR luciferase Plasmids (Figure 2B). Western Blot assay showed, comparing to the result in scramble group, the expression of Wnt2 was significantly inhibited in U252 cells after transfected with miR-199a-5p mimic (Figure 2C, P < 0.01). The above experiments proved that the miR-199a-5p target-inhibited Wnt2 expression in glioma cells.
miR-199a-5p over-expressed lentivirus inhibited the activation of Wnt2/β-catenin signaling pathway in U251 cells

Compared with negative control of lentivirus infected U251 cells, the expression of miR-199a-5p in miR-199a-OE LV infected U251 cells was significantly increased (Figure 3A, P < 0.001), while the expression of Wnt2 and β-catenin was significantly inhibited (Figure 3B, P < 0.001, P < 0.001).

miR-199a-5p inhibited the proliferation of U251 cells

Compared with negative control of lentiviral infections group, the proliferation of U251 cells was significantly inhibited (Figure 4A, P < 0.001) and the expression of c-MYC and cyclinD1 in U251 cells was significantly down-regulated (Figure 4B, P < 0.001, P < 0.001) in miR-199a-OE LV infected U251 cells.

MiR-199a-5p promoted the apoptosis of U251 cells

Compared with negative control of lentiviral infections group, the apoptosis rate of U251 cells was significantly increased (Figure 5A, P < 0.001) and the expression of Bax, Bad and caspase3 in U251 cells was significantly up-regulated (Figure 5B, P < 0.001) after miR-199a-OE LV infection.

MiR-199a-5p inhibited the invasion of U251 cells

Compared with negative control of lentiviral infections group, the invasive ability of U251
cells decreased after miR-199a-OE LV infection (Figure 6A, P < 0.01). The expression of MMP-2 and MMP9 in U251 cells also significantly decreased after miR-199a-OE LV infection (Figure 6B, P < 0.001).

Discussion

Brain stem gliomas more likely to happen on children, and the prognosis is poor. At present, the main treatment methods are chemical
treatment, radiation therapy and surgery, but the three methods have their own shortcomings [8]. Various chemotherapy regimens and drugs can not significantly improve the prognosis while radiation therapy can only temporarily alleviate the symptoms of patients with unsatisfying prognosis. And the risk of surgical treatment is high, and many patients do not meet the conditions of surgical treatment [9].

The clinical treatment effect of glioma differs from each other since the tumor location differs. For example, the prognosis of brain stem glioma is worse than cerebral hemisphere glioma. We speculate that it might be related with the importance of brainstem as the vital center of the physiological function etc. In addition, the current study of brain stem glioma is far from the research of cerebral hemisphere glioma [10, 11]. The treatment effect of brain stem glioma and cerebral hemisphere glioma differs a lot after treated with same treatment, suggesting that the development mechanism of the two may be different. Further exploring the pathological and physiological mechanism of brainstem glioma to find a more effective treatment is currently the main research trend [12-14].

Wnt/β-catenin signaling pathway, involving in embryonic development, stem cell differentiation, energy metabolism and other multiple physiological processes, is highly conserved in its evolution. Wnt/β-catenin signaling pathway plays an important role in the development and progression of a variety of tumors, including brain glioma [15]. The researches show that there is an abnormal activation of Wnt/β-catenin signaling pathway in brain glioma, but the modulate mode of the activation is not very clear.

In the Wnt/β-catenin signaling pathway, Wnt is the initiation protein and plays an important role in the aspects like cell polarity, proliferation, migration, and differentiation etc [16]. This study showed that the Wnt-2 expressed in both brainstem glioma tissues and normal brain tissues, but its expression in normal brain tissues and low-grade brainstem glioma were significantly lower than that in high-grade brainstem glioma, suggesting there might be a molecular mechanism in brainstem glioma that activate the Wnt2/β-catenin signaling pathway by promoting the expression of Wnt2, thus involve in the occurrence and progress of tumor.

The present study has demonstrated that microRNA involved in the regulation of many tumors. Bioinformatics analysis of Wnt2 revealed that miR-199a-5p might involved in the regulation of Wnt2 expression; the detection of miR-199a-5p expression showed that miR-199a-5p expression was significantly lower in glioma tissues than normal brain tissues and the expression was negatively correlated with the malignant degree of brain stem glioma. Thus we speculated that MiR-199a-5p target-inhibited the expression of Wnt2 in U251 glioma cells, which was then proved by luciferase assay and Western Blot assay.

We used the lentivirus-mediated method to raise the expression of miR-199a-5p in U251 cells. Cell proliferation and apoptosis analysis showed that miR-199a-5p can significantly inhibit the proliferation of U251 cells and promoted its apoptosis; Western Blot showed that c-MYC and cyclinD1 were inhibited, while the expression of Bax, Bad and caspase3 were significantly increased in U251 cells. Traswell assay showed that miR-199a-5p significantly inhibited the metastasis of U251 cells; while Western Blot showed MMP2 and MMP9 were inhibited in the same time. Functional analysis of miR-199a-5p in U251 cells demonstrated that miR-199a-5p plays a role as tumor gene suppressor by inhibiting Wnt2/β-catenin signaling pathway to participate in the regulation of the ability in cell proliferation cycle, apoptosis and metastasis.

This study showed that there was an abnormal activation of Wnt2/β-catenin signaling pathway in brainstem glioma, which involved in the occurrence and development of brainstem glioma. Our further study revealed that the expression deletion of miR-199a-5p was an important cause of abnormal activation of Wnt2/β-catenin signaling pathway in glioma, and it involved in the occurrence and development of brainstem glioma. The modulation mode of miR-199a-5p expression in brain stem glioma and its value as a potential prognostic marker for brain stem glioma should be further studied.

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Study on the Wnt/β-catenin signaling pathway

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Disclosure of conflict of interest
None.

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