Original Article

MiR-9 promotes proliferation of glioma cells by regulating Foxo3a-p27kip1 signaling axis

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Abstract: FoxO3a is a well-defined tumor suppressor gene and its expression is associated with multiple tumorigenesis events. Elevated miR-9 level was found in glioma tissues. Bioinformatics analysis revealed a targeted complementary relationship between miR-9 and FoxO3a. This study investigated the role of miR-9 in glioma cell proliferation and apoptosis. Tumor tissues and para-cancerous tissues were collected from glioma patients. qRT-PCR was used to detect miR-9 and Foxo3a mRNA expression. The dual luciferase reporter gene assay validated the targeted regulation between miR-9 and Foxo3a. Glioma Hs683 cells were cultured in vitro and divided into two groups: miR-NC group and miR-9 inhibitor group, followed by analysis of miR-9 and Foxo3a mRNA levels, Foxo3a and p27Kip1 protein expression, cell apoptosis by flow cytometry, and cell proliferation by EdU staining. Compared with that in adjacent tissues, miR-9 expression in glioma tissues was significantly increased, and Foxo3a mRNA expression was significantly decreased. The dual luciferase reporter gene experiment confirmed that there was a targeted regulation relationship between miR-9 and Foxo3a. Transfection of miR-9 inhibitor significantly inhibited miR-9 expression in Hs683 cells, increased the expression of Foxo3a and p27Kip1, decreased cell proliferation, and increased cell apoptosis. In conclusion, elevated miR-9 expression plays a role in down-regulating Foxo3a expression and promoting the pathogenesis of glioma. Down-regulation of miR-9 expression can increase Foxo3a expression, inhibit glioma cell proliferation, and promote cell apoptosis.

Keywords: miR-9, Foxo3a, glioma, proliferation, apoptosis

Introduction

Glioma is one of the most common intracranial central nervous system malignancies. It has a high degree of malignancy and rapid disease progression, posing a serious threat to patients' lives and health [1].

FoxO3a is a member of the forkhead transcription factor O subfamily (FoxO) and a relatively clear tumor suppressor gene [2-4]. Foxo3a can regulate the transcription and expression of a variety of genes of interest, thereby affecting the biological processes of proliferation, apoptosis, migration and invasion of tumor cells [5-8]. A number of studies have shown that the abnormal expression or dysfunction of Foxo3a is closely related to the occurrence, progression, metastasis and drug resistance of various tumors [6, 9, 10]. Foxo3a plays a role in regulating the proliferation and apoptosis of glioma cells, and the expression or functional activity of Foxo3a is related to the occurrence, progression and prognosis of gliomas [11-13].

MicroRNA (microRNA) is a newly discovered non-coding single-stranded small RNA molecule with a length of 19-25 nucleotides. It is an extremely important gene regulatory factor and regulates the expression of gene mRNA through complementary binding to the 3'-untranslated region (3'-UTR), leading to degradation of the target mRNA or inhibition of post-transcriptional translation, thereby participating in the regulation of biological processes such as cell growth, differentiation, apoptosis, and migration [14, 15]. Studies have shown that abnormally elevated expression of miR-9 is associated with the development and progression of glioma, suggesting that miR-9 might be a cancer-promoting factor in the pathogenesis of glioma [16, 17]. Bioinformatics analysis revealed
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a targeted complementary binding site between miR-9 and the 3'-UTR of Foxo3a mRNA. This study investigated whether miR-9 plays a role in regulating Foxo3a expression, affecting glioma cell proliferation and apoptosis.

Materials and methods

Main reagents and materials

Human glioma Hs683 and SHG44 cells were purchased from Hunan Fenghui organism; HEK293T cells and human normal glial HEB cells were purchased from Shanghai Yaji Bio; DMEM medium, fetal bovine serum (FBS), Opti-MEM reduced serum medium was purchased from the United States Gibco; ReverTra Ace qPCR RT Kit, SYBR dye was purchased from Japan Toyobo; miR-NC, miR-9 mimic, miR-9 inhibitor were purchased from Guangzhou Ruibo biological; rabbit anti-human Foxo3a, p27Kip1 monoclonal antibody were purchased in the United States from Abcam; Rabbit anti-human β-actin antibody and HRP-conjugated goat anti-rabbit secondary antibody were purchased from Shanghai Shenggong Bio; BCA protein quantification kit was purchased from Beijing Solarbio; CCK-8 reagent and Annexin V/PI apoptosis detection reagent were purchased from Jiangsu Biyuntian Bio; Dual-Luciferase Reporter Assay System was purchased from Promega (USA); pMIR plasmid was purchased from Changsha Youbao Bio; EdU Cell Proliferation Assay Kit was purchased from Molecular Probes (USA).

Patients

A total of 38 patients with glioma who were treated in our hospital from March 2018 to September 2018 were enrolled. The specimens of the tumor tissue which was removed during operation were collected, and the paracancerous tissues which were located 2 cm around the tumors were collected as controls. The physical examination confirmed the diagnosis. All tissue specimens were collected in liquid nitrogen and then stored in a -80°C freezer. This study was approved by the ethnics committee of our hospital and informed consents was obtained from all patients.

Cell culture

HEB, Hs683 and SHG44 cells were cultured in DMEM medium containing 10% FBS and 1% streptomycin in a cell culture incubator containing 5% CO₂ at 37°C. After cells reached a confluence of 70-80%, 0.125% trypsin was used to digest the cells, which were then subcultured at a ratio of 1:5 to 1:6, and the experiment was performed while the cells were in a log phase of good cell growth.

Dual luciferase gene reporter assay

The mRNA of HEK293T cells was extracted using Trizol reagent, and the fragment containing the targeted binding site or its mutant fragment in the 3'-UTR region of Foxo3a gene was amplified using mRNA as a template. The PCR product was recovered by gelatinization and digested into pMIR vector. DH5α competent cells were transformed, positive clones were screened by colony PCR, and the correct plasmids were selected and named as pMIR-Foxo3a-WT and pMIR-Foxo3a-MUT, respectively.

pMIR-Foxo3a-WT (or pMIR-Foxo3a-MUT) and miR-9 mimic (or miR-NC) were co-transfected into HEK293T cells with Lip 2000. After 48 h of culture, the relative luciferase activity was measured using the Reporter Assay System kit according to the instructions of the kit.

Cell transfection and grouping

SHG44 cells were cultured in vitro and divided into two groups: miR-NC transfection group, and miR-9 inhibitor transfection group. The transfection procedure was performed as follows: 10 μL of Lip 2000 and 50 nmoL miR-NC were diluted with 100 μL of serum-free Opti-MEM. Then 50 nmoL miR-9 inhibitor was incubated for 5 min at room temperature. Lip 2000 was gently mixed with miR-NC and miR-9 inhibitor, and incubated at room temperature for 25 min. The transfectants were separately added to the cell culture medium. After gently mixing, the culture was continued for 72 hours, and the cells were collected.

Flow detection of cell apoptosis

The two transfected cell lines were collected after trypsinization. After washing twice with PBS, 100 μL of Binding Buffer was added to the cell pellet, and 5 μL of Annexin V-FITC and 5 μL of PI were added by pipetting. The reaction was incubated for 20 min and 400 μL of Binding buffer was added followed by analysis of cell apoptosis by Beckman Coulter FC 500 MCL flow cytometry.
**Flow detection of cell proliferation**

The two transfected cell lines were harvested after trypsinization and resuspended in RPMI 1640 complete medium containing 10% FBS. After incubation with 10 μM of EdU at 37°C for 2 h, the cells were further cultured for 48 h. The cells were washed twice with PBS and fixed by 100 μL. The solution was fixed at room temperature for 15 min, centrifuged in PBS, and then incubated with 100 μL permeabilization solution for 15 min at room temperature. Then add 500 μL of the reaction solution, incubate at room temperature for 30 min in the dark, add 3 mL of washing solution, centrifuge once, and resuspend the cells in 500 μL of washing solution. After that, cell proliferation was detected by flow cytometry.

**qRT-PCR detection of gene expression**

RNA was extracted using Trizol reagent, and reversely transcribed into cDNA using ReverTra Ace qPCR RT Kit. The qRT-PCR was performed in a 20 μL system including: 2 μg total RNA, 1 μL dNTP, 4 μL Buffer, 1 μL RT primer, 2 μL RT Enzyme, 1 μL RNase inhibitor, and ddH2O. The PCR amplification reaction conditions were: 95°C for 15 s, 60°C for 30 s, and 74°C for 30 s, and data were collected after 40 cycles of amplification on a Bio-Rad CFX96 real-time PCR machine.

**Western blot**

We added 100 μL of RIPA protein extract to every 5 mg or 1 million cells, fully lysed at 4°C, centrifuged at 10,000 g for 15 min, transferred the protein supernatant to a new EP tube, followed by measurement of the mass concentration by BCA. Fifty μg protein was separated on 10% SDS-PAGE gel, transferred to PVDF membrane, blocked with 5% skim milk powder, and incubated with the primary antibody (Foxo3a, p27Kip1, and β-actin were diluted 1:500, 1:1, 000, 1:5,000, respectively) overnight at 4°C. After washing 3 times with PBST, HRP-labeled goat anti-rabbit secondary antibody (1:5,000 dilution) was incubated with the membrane for 1 h at room temperature. Wash with PBST 3 times, add ECL luminescent solution for 1~3 min, expose, develop, fix, scan the film and save the data.

**Statistical analysis**

Statistical analysis was performed using SPSS 18.0 software. The measurement data were expressed as mean ± standard deviation (SD). The student’s t test was used to compare the measurement data between the two groups. The comparison between the measurement data of multiple groups was analyzed by one-way ANOVA with Binferroni post-hoc analysis. The Mann-Whitney U test was used to compare the expression levels of miR-9 and Foxo3a mRNA. P<0.05 was considered statistically significant.

**Results**

**Increased miR-9 expression and decreased Foxo3a expression in glioma tissues**

The results of qRT-PCR showed that the expression of miR-9 was significantly increased in tumor tissues of glioma patients compared with adjacent tissues (Figure 1A). Meanwhile,
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the expression of Foxo3a mRNA in tumor tissues of glioma patients was significantly decreased compared with adjacent tissues (Figure 1B). This data suggests that miR-9 and Foxo3a might be involved in the pathogenesis of gliomas.

Targeted regulation between miR-9 and Foxo3a

Bioinformatics analysis revealed a targeted complementary binding site between miR-9 and the 3’-UTR of Foxo3a mRNA (Figure 2A). The dual luciferase gene reporter assay showed that transfection of miR-9 mimic significantly reduced the relative luciferase activity of pMIR-Foxo3a-WT-transfected HEK293T cells, but miR-9 mimic or miR-NC did not have significant effects on relative luciferase activity in pMIR-Foxo3a-MUT-transfected HEK293T cells (Figure 2B), confirming that there was a targeted regulatory relationship between miR-9 and Foxo3a mRNA.

Increased miR-9 expression and decreased Foxo3a expression in glioma

The results of qRT-PCR showed that the expression of Foxo3a mRNA in glioma Hs683 and SHG44 cells was significantly lower than that in normal glial HEB cells (Figure 3B). Western blot analysis showed that the expression of Foxo3a protein in glioma Hs683 and SHG44 cells was significantly lower than that in normal glial HEB cells (Figure 3C).

Inhibition of miR-9 expression promotes apoptosis of glioma cells and attenuates cell proliferation

The results of qRT-PCR showed that the expression of miR-9 in Hs683 cells was significantly decreased in miR-9 inhibitor transfected group compared with miR-NC group (Figure 4A), while the expression of Foxo3a mRNA was significantly increased (Figure 4B). Western blot analysis showed that the expression of Foxo3a and p27Kip1 protein in Hs683 cells was significantly increased in miR-9 inhibitor transfected group compared with miR-NC group (Figure 4C). Flow cytometry analysis showed that the apoptosis of Hs683 cells in the miR-9 inhibitor transfection group was significantly increased compared with the miR-NC group (Figure 4D), and the proliferative ability was significantly inhibited (Figure 4E). Taken together, miR-9 plays a role in the regulation of proliferation and apoptosis of glioma cells.

Discussion

Glioma has the characteristics of high malignancy, strong invasiveness, high recurrence and metastasis rate. The existing surgical resection, radiotherapy and chemotherapy, immunotherapy and other approaches have not achieved satisfactory results, and the survival and prognosis are extremely poor. It has the worst prognosis of malignant tumors, with a 5-year survival rate of less than 5% [18, 19]. Therefore, studying the pathogenesis of glioma and exploring the signal-regulating molecules of abnormal changes in the pathogenesis of glioma are of great significance to improve the diagnosis, therapeutic effect as well as prognosis.

The FoxO transcription factor family contains four members, FoxO1, FoxO3a, FoxO4, and FoxO6, of which FoxO3a is the most studied member [2-4]. FoxO3a is a relatively clear tumor suppressor gene [2-4]. As a transcriptional regulator, FoxO3a regulates the transcriptional expression of various genes such as BIM, PUMA, p27Kip1, and cyclin D1, thereby...
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Figure 3. Increased expression of miR-9 in gliomas and decreased expression of Foxo3a. A. qRT-PCR detection of Foxo3a mRNA expression in glioma cells; B. qRT-PCR detection of miR-9 expression in glioma cells; C. Western blot analysis of Foxo3a protein expression in glioma cells. *Represents P<0.05 compared to HEB cells.

Figure 4. Inhibition of miR-9 expression promotes glioma cell apoptosis and attenuates cell proliferation. A. qRT-PCR was used to detect the expression of miR-155 in hepatoma cells; B. qRT-PCR was used to detect the expression of Foxo3a mRNA in hepatoma cells; C. Western blot was used to detect the expression of Foxo3a and p27Kip1 proteins in hepatoma cells; D. Flow cytometry detection of glioma cell apoptosis; E. Flow cytometric detection of glioma cell proliferation. *Represents P<0.05 compared to the miR-NC group.

regulating various biological processes such as cell proliferation, apoptosis, cycle, invasion and metastasis [20-22]. A number of studies have shown that the abnormal expression or function of Foxo3a is closely related to the occurrence, progression, metastasis and drug resistance of various tumors such as prostate cancer [6], ovarian cancer [9], breast cancer [10]. Studies have found that decreased expression or functional activity of Foxo3a is associated with the development, progression, and prognosis of glioma [11-13].

Studies have shown that abnormal expression of miR-9 plays a role in the occurrence, progression and metastasis of various tumors such as prostate cancer [23] and non-small cell lung cancer [24]. Abnormally elevated expression of miR-9 is associated with the development and progression of glioma, suggesting that miR-9 is a cancer-promoting factor in the pathogenesis of glioma [16, 17]. This study investigated whether miR-9 plays a role in regulating Foxo3a expression, affecting glioma cell proliferation and apoptosis.

In this study, the dual luciferase gene reporter assay showed that transfection of miR-9 mimic significantly reduced the relative luciferase activity of pCMV-Foxo3a-WT-transfected HEK293T
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cells, whereas, miR-NC and miR-9 mimic did not have significant effects on the relative luciferase activity of HEK293T cells transfected with pMIR-Foxo3a-MUT, confirming that there was a targeted regulation between miR-9 and Foxo3a. The results of clinical samples showed that compared with the adjacent tissues, the expression of miR-9 in the tumor tissues of glioma patients was significantly increased, while the expression of Foxo3a mRNA and protein was significantly decreased, suggesting that the expression of miR-9 was abnormally elevated and may play a role in inhibiting Foxo3a expression and promoting the pathogenesis of glioma. The results of cell culture in vitro showed that compared with normal glial HEB cells, the expression of miR-9 in glioma Hs683 and SHG44 cells was significantly increased, while the expression of Foxo3a was significantly decreased, further suggesting increased miR-9 expression and decreased expression of Foxo3a may play a role in gliomas. In the study of the relationship between miR-9 and glioma, Chen et al. [16] showed that the expression of miR-9 in peripheral blood serum of patients with glioma was significantly higher than that of healthy controls. The results of Katakowski et al. [25] showed that the expression of miR-9 was significantly increased in the lesion center of glioma metastasis compared with the lesions surrounding the tumor. Malzkorn et al. [26] showed that the expression of miR-9 in relapsed and progressive glioma tissues was significantly increased, ranging from 3.16 to 5.03 times, compared with primary glioma. The results of Wu et al. [17] showed that compared with non-neoplastic brain tissue, the expression of miR-9 in tumor tissues of glioma patients was significantly increased, and the higher pathological grade of glioma, the higher expression of miR-9; the worse prognosis of patients and the lower survival rate. The results of Ye et al. [27] showed that the expression of miR-9 was abnormally elevated in tumor tissues of glioma patients compared with normal brain tissue. In this study, the expression of miR-9 in glioma tissues and glioma cells was abnormally elevated, and the increase of miR-9 expression may play a role in cancer promotion in gliomas, consistent with Katakowski et al. [25], Malzkorn [26], and Wu et al. [17].

p27Kip1 is one of the negative regulators of cell cycle, and its expression changes with the change of cell cycle progression. Through arresting cells in G1 phase, it plays an important role in regulating cell cycle progression, so that cell proliferation is inhibited [28, 29]. In this study, miR-9 inhibitor was transfected into glioma cells cultured in vitro, and the changes in cell biological effects were observed. The results showed that transfection of miR-9 inhibitor significantly increased the expression of Foxo3a and p27Kip1 in glioma Hs683 cells, which significantly attenuated the proliferation of cells and significantly increased apoptosis, indicating that miR-9 plays a role in promoting the proliferation of glioma cells and antagonizing apoptosis through targeting Foxo3a. In the study of miR-9 regulating the biological effects of glioma cells, Chen et al. [16] showed that increasing the expression of miR-9 can promote the proliferation of glioma cells and enhance the migration and invasion ability of glioma cells. MiR-9 can also promote angiogenesis in glioma by targeting inhibition of COL18A1, THBS2, PTCH1, PHD3 expression, and play a role in cancer promotion in glioma. Katakowski et al. [25] showed that in the low-density culture, overexpression of miR-9 in glioma 9L and U87 cells significantly promoted the proliferation of glioma cells and enhanced the invasive ability of cells. Our present study combines the targeting relationship between miR-9 and Foxo3a, revealing that elevated expression of miR-9 plays a role in down-regulating Foxo3a expression and promoting the pathogenesis of glioma, which has not been reported in previous studies. However, whether targeting the regulation between miR9 and Foxo3a in existing glioma patients can have therapeutic effects remains to be confirmed by further studies.

Conclusion

The elevated expression of miR-9 plays a role in down-regulating the expression of Foxo3a and promotes the pathogenesis of glioma. Down-regulation of miR-9 expression can increase Foxo3a expression, inhibit glioma cell proliferation, and promote cell apoptosis.

Disclosure of conflict of interest

None.

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