The Expression of miR-21 in Brain Glioma Cells and its Effect of PI3K/AKT Signal Pathway Running

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ABSTRACT

Background: Brain glioma is a common primary intracranial tumor with unfavorable prognosis. MicroRNA-21 (miR-21) is a small RNA molecule involved in post-transcriptional modulation and is closely associated with cell growth and differentiation. Recent studies have shown that miR-21 expression affects tumor proliferation and growth via the PI3K/AKT signaling pathway. This study investigated the expression of miR-21 in glioma and its effects on the PI3K/AKT pathway and cell proliferation.

Methods: A total of 35 brain glioma patients were recruited from March 2013 to November 2014 at our hospital. In situ hybridization (ISH) and RT-PCR were used to detect the expression of miR-21 in both tumors and adjacent brain tissues. Human brain U251 glioma cells were transfected with anti-miR-21 to suppress miR-21 expression, followed by immunofluorescent assay of PI3K and AKT expression profiles. Flow cytometry was employed to detect the change between different stages of the cell cycle.

Results: ISH results showed significantly elevated miR-21 expression level in glioma tissues compared to normal brain tissues. RT-PCR analysis revealed a 3.5-fold increase in miR-21 in the anti-miR-21 transfected glioma cells compared to untransfected cells. After silencing miR-21 in U251 cells, immunofluorescent assay indicated that the expression of PI3K and AKT significantly decreased. Flow cytometry revealed an increased number of cells at G0/G1 phase in anti-miR-21 transfected U251 cells, suggesting suppressed cell division ability.

Conclusion: MiR-21 expression was found to be increased in brain glioma tissues, accompanied with over-activation of PI3K/AKT signaling and proliferation of glioma cells.

Key Words: Brain glioma; MicroRNA-21; PI3K/AKT signal pathway; Cell proliferation

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INTRODUCTION

Brain glioma is a common primary intracranial tumor with high

malignancy [1] . Current research indicates that brain glioma is caused by a combination of genetic predisposing factors and oncogenic environmental factors [2]. Although a combined therapy of neurosurgery and chemo-, radio-, immuno- and molecular targeted therapy can significantly prolong patients' life and improve their quality of life[3,4], brain glioma has a high recurrence rate and patients with brain glioma have poor prognosis due to its rapid progression, high heterogeneity, and diffuse infiltrative growth characteristics [5]. Therefore, a study of the pathogenesis and molecular biology of brain gliomas is critical for further improving treatment efficacy and extending patients' survival.

MicroRNAs (miRNAs) are small, highly conserved, noncoding RNAs that regulate gene expression by binding to complementary sites on target transcripts and are important modulators of pathophysiology processes [6,7]. MicroRNA-21 (MiR-21) is involved in modulating multiple biological behaviors including cell growth, division and differentiation [8]. Interestingly, changed miR-21 levels have been found in various malignant tumors and linked to drug-resistance of tumor cells [9]. PI3K is best-characterized direct target of miR-21 [10]. A subsequent study found that the effects of miR-21 on tumor cells are mediated by modulating the PI3K/AKT signaling pathway [10]. Thus, the study investigated the expression of miR-21 in brain glioma cells and its correlation with PI3K/AKT signaling.

MATERIALS AND METHODS

Research Subjects

A total of 35 patients with brain glioma (20 males and 15 females, aged from 28 to 64 years, average age = 39.1 ± 6.5 years) were recruited for this study from December 2013 to December 2014 at our hospital. 14 brain tissues were collected from internal decompression after severe brain trauma (8 males, 6 females, aging between 31 and 67 years, average age = 42.3 ± 8.2 years) as the control group. All glioma patients had not received radio-/chemo-/immune-therapy before admission. The lesion was confirmed by head MRI.

In Situ Hybridization (ISH)

Formalin fixed paraffin embedded (FFPE) tissues were sectioned at 4 μm for ISH assay. DIG (digoxigenin) labeled anti-sense miR-21 oligonucleotide probe (5'- TCAACATCAG-TCTGATAAGCTA -3') was purchased from Boster (China) and used as the probe in the ISH assay as previously described [11]. Briefly, the FFPE sections were deparaffinized, rehydrated, and blocked for peroxidase activity, followed by proteinase K digestion and PBS (0.5M, pH 7.4) rinsing. Pre-hybridization was performed for 3~4 hours at 63°C~65°C, followed by the addition of hybridization buffer containing the probe for 12~16 hours at 65°C. After hybridization, 0.2XSSC solution was used to wash the sections. After blocking at room temperature, the sections were incubated with anti-rabbit DIG antibody for 1-hour at 37°C. After rinsing with PBS, alkaline phosphatase was added, followed by color development using alkaline phosphatase substrates. The reaction was quenched by PBS washing. The sections were fixed in 4% paraformaldehyde for 2 hours, followed by PBS rinsing and cover slice mounting. The sections were observed under a bright field microscope.

RT-PCR

MicroRNA was extracted from FFPE tissues using an extraction kit from Baitaike, China. Normal brain tissues were used as the control. RT-PCR was performed using the mirVanat qRT-PCR miRNA test kit (Ambion, USA) on a real-time PCR cycler (Bio-Rad) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C, 15 sec and 60 °C, 30 sec. Results were analyzed using 2- △ △ Ct method as previously reported [12].

Cell Transfection

Anti-miR-21 and negative control nucleotide sequence with methylation modification were synthesized by Sangon (China) as listed in Table 1. Transfection was performed using Lipofectome INTERFERin transfection kit (Polyplus transfection). Log-phase grown brain glioma U251 cells (Cell Bank, Chinese Academy of Sciences)were detached with trypsin. The cells were counted, properly diluted and seeded into 96-well plates. After culturing for 24-hourse, cells were transfected with anti-miR-21 and negative control oligos respectively, using the lipofection method according to the instructions of the transfection kit [13].

Immunofluorescent Assay

FFPE sections were de-waxed and re-hydrated, followed by antigen retrieval and blocking in 0.5% bovine serum albumin. Sections were incubated with mouse anti-PI3K or anti-AKT antibody overnight at 4°C, followed by incubation with FITC fluorescent labeled goat anti-mouse antibody for 30 min at room temperature after washing. After adding anti-fade reagents, sections were covered with coverslips. Images were captured under a fluorescent microscope [14].

Flow Cytometry

Flow cytometry was used to detect cell cycle stage. Transfected U251 cells were cultured to log-phase growth. Cells were harvested and centrifuged. After washing with 1 × PBS, cells were fixed in 90% ethanol at 4 °C overnight. After discarding ethanol, cells were incubated with RNase for 30-min at 37 °C, followed by staining with PI (propidium iodide). Cells were loaded onto the flow cytometer (Becton Dickinson) and sorted at 488nm excitation / 630 nm emission wavelength. Ten thousand fluorescent signals were collected to plot FL-2 area and DNA histogram by Modifit software. All experiments were replicated for three times, and presented as the percentage of cells at specific cycles [15].

STATISTICAL ANALYSIS

SPSS 17.0 software was used to process all collected data, which were presented as mean ± standard deviation (SD). The comparison between groups- was performed by student t-test. Multiple group comparison was done by the analysis of variance (ANOVA), followed by q-test. A statistical significance was defined when $p<0.05$.

RESULTS

MiR-21 Expression in Glioma and Normal Brain Tissues

We performed an ISH study in glioma and normal brain tissues using a miR-21 specific probe. miR-21 was mainly expressed in the cytoplasm, shown as blue-violet granules in ISH (Fig. 1). The intensity of staining reflects relative expression of miR-12. The staining intensity was higher in glioma tissues than in normal brain tissues, suggesting a higher expression level of miR-21 in brain glioma cells. The ISH results were further confirmed in the RT-PCR study (Fig. 2), which showed significantly elevated expression of miR-21 in glioma tissues compared to normal brain tissues.

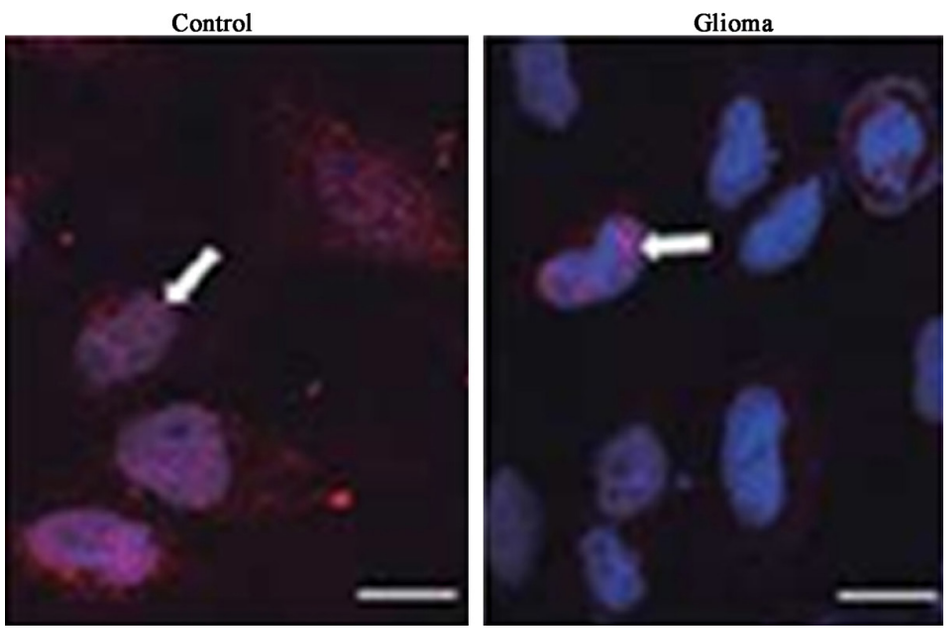

Fig. 1 miR-21 expression in glioma and normal brain tissues were detected by ISH. FFPE sections were deparaffinized.The miR-21 transcripts were detected by ISH using DIG labeled miR-21 specific antisense oligo. The miR-21 level shown as blue-violet granules was visualized by alkaline phosphatase catalyzed color development. Increased the blue-violet granules in glioma tissues indicate miR-21 expression.

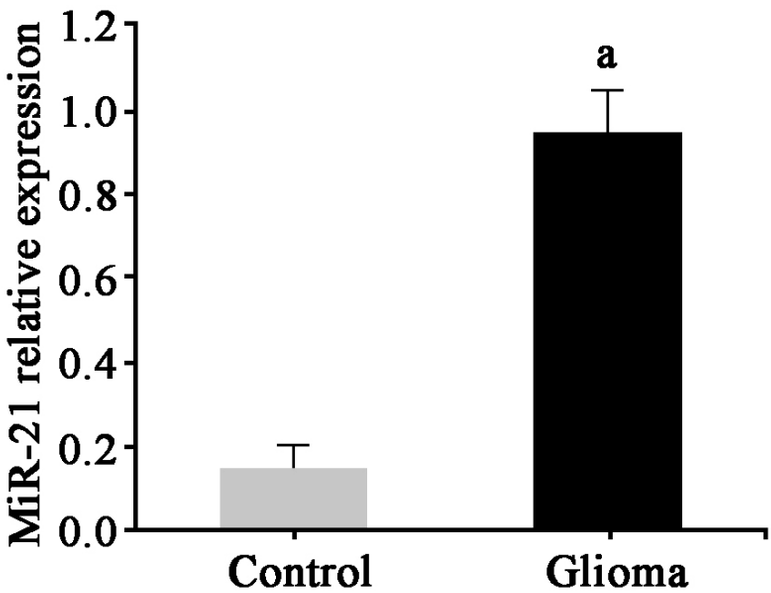

Fig. 2 miR-21 expression in glioma and normal brain tissues were detected by real-time PCR. a $p<0.01$ compared to control group.

MiR-21 Expression after Transfecting Anti-miR-21 in U251 Cells

We further used anti-miR-21 (an antisense oligo of matured miR-21) to transfect U251 brain glioma cells, followed by RT-PCR to quantify miR-21 expression. Using ck-siRNA as the control, we found the relative expression level of miR-21 was 0.61 ± 0.051, 0.93 ± 0.11 and 0.96 ± 0.12 for anti-miR-21 transfected cells, ck-siRNA control cells, and blank controls. The level of miR-21 in anti-miR-21 transfected cells was significantly lower than that in blank control and ck-siRNA control ($p<0.05$,Fig. 3) cells, suggesting the successfully silencing of miR-21 expression.

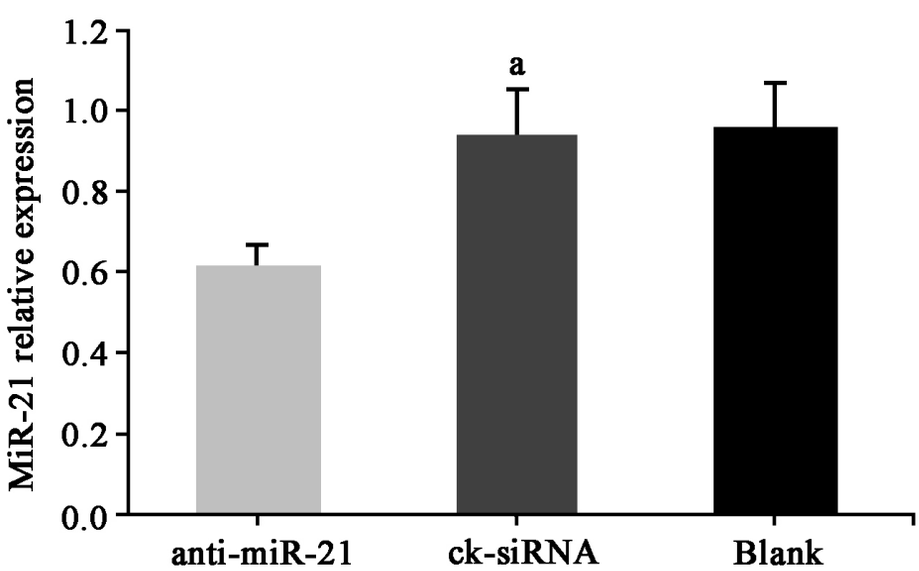

Fig. 3 Inhibition of MiR-21 expression in U251 cells with anti-miRNA oligo. U251 glioma cells were transfected with anti-miR-21 oligos (anti-miR-21), negative control oligos (ck-siRNA), and no oligo. miR-21 expression was measured using a real-time PCR. ap<0.05 compared to control group.

PI3K and AKT Expression

Cells transfected with anti-miR-21 were fixed and stained using specific antibodies against PI3K and AKT followed by fluorescence labeled secondary antibody. As shown in Fig. 4, PI3K and AKT were expressed in the cytoplasm. The cells with green fluorescence indicate positive expression for PI3K and AKT proteins. We found significantly decreased intensity of green fluorescence in the anti-miR-21 transfected cells, suggesting lower PI3K and AKT protein expression.

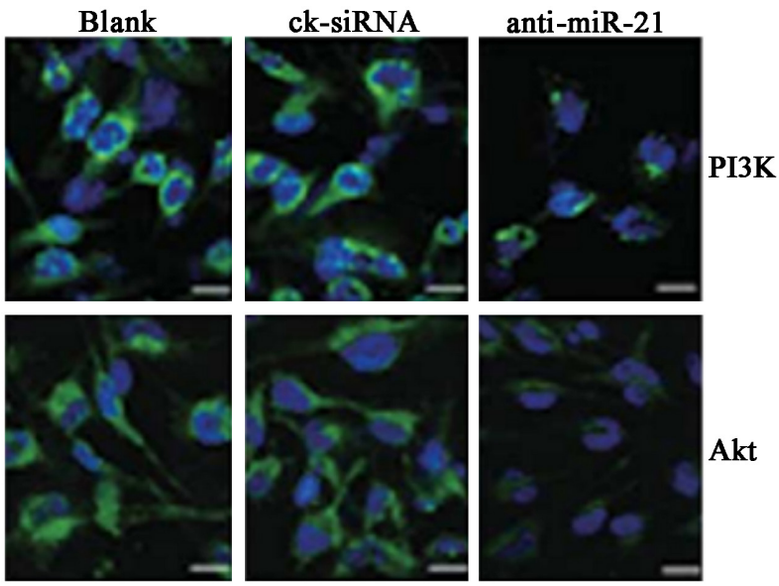

Fig. 4 PI3K and AKT protein expression. U251 glioma cells were transfected with anti-miR-21 oligos (anti-miR-21), negative control oligos (ck-siRNA), and no oligos. PI3K and Akt expression was detected by immunofluorescent staining. Obvious reduced intensity of green fluorescence was observed in cells transfected with anti-miR-21.

Cell Cycle Alternation

We further employed flow cytometry to determine the cell cycle stage of the miR-21 antisense oligo transfected cells and control cells. Modifit software generated FL-2 area and DNA histogram were shown in Fig. 5. The statistical analysis of cell numbers at different cycles was shown in Fig. 6. No significant difference existed in cell number at G0/G1, S and G2/M phase between blank and negative control groups ($p>0.05$). Anti-miR-21 transfected group had significantly increased number of G0/G1 cells ($p<0.05$) and lowered number of S or G2/M cells ($p<0.05$), suggesting extended cell cycle length and impaired mitosis ability.

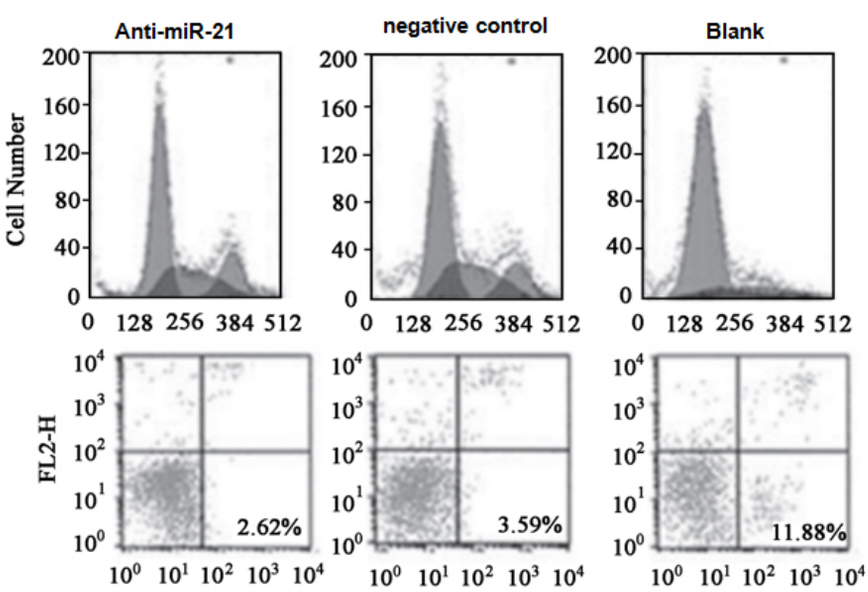

Fig. 5

Fig. 5 Flow cytometry of cell cycle. U251 glioma cells were transfected with anti-miR-21 oligos (anti-miR-21), negative control oligos (ck-siRNA), and no oligos. Representative flow cytometry shows the percentage of G0/G1, S or G2/M cells.

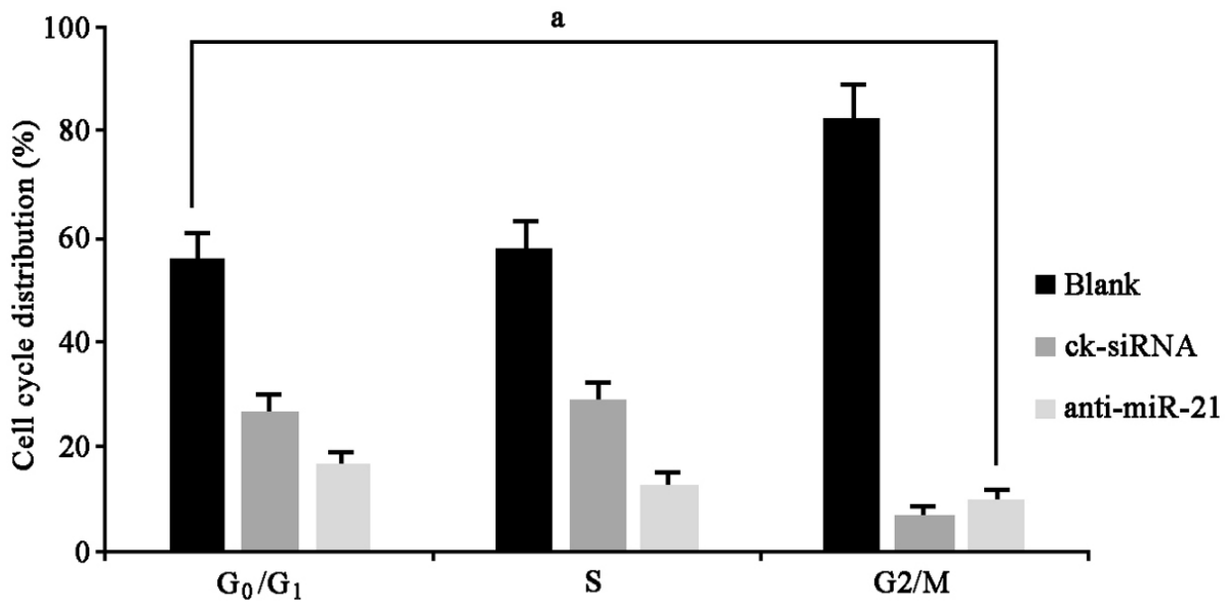

Fig. 6 Cell cycle analysis in anti-miR-21-transfected U251 cells. Significantly increased number of G0/G1 cells and lowered number of S or G2/M cells was observed in anti-miR-21 transfected U251 cells. ap<0.05 compared to control group.

DISCUSSION

Brain glioma is a common primary intracranial tumor with unfavorable prognosis [16]. Recent studies have shown that miR-21 expression affects tumor proliferation [17]. In our study, the results of ISH analysis and RT-PCR revealed an elevated expression of miR-21 in tumor cells compared to normal brain tissues. Current research indicates that microRNA participate in the modulation of cell proliferation, growth, and differentiation [18]. This study demonstrated that inactivation of miR-21 expression significantly increased number of cells in the G0/G1 stage and lowered the number of cells in the S or G2/M stage, suggesting extended cell cycle length and impaired mitosis ability. Thus, miR-21 represents a potential therapeutic target for the treatment of brain glioma

PI3k/AKT pathway not only plays an important role in regulating insulin sugar metabolism, but also promotes cell survival and apoptosis, and the deformation and movement of nerve cells [19-21]. In addition, PI3k/AKT pathway was reported to be regulated by PTEN protein in maintaining cell proliferation and division at normal levels [22]. Interestingly, PTEN was reported to be significantly inhibited by miR-21 through binding to the 3'-UTR of PTEN mRNA in the liver cancer cells [19, 23] Consistent with these observations, we found that inactivation of miR-21 through the anti-sense oligo of miR-21 significantly decreased PI3K and Akt expression in glioma cells. The results revealed that PI3k/AKT pathway can be regulated by miR-21 and the effects of miR-21 on glioma was involved in the regulating PI3k/AKT pathway.A previous study showed that silencing of microRNA-21 can increase radio-sensitivity in malignant glioma cell lines through inhibition of the PI3K/AKT pathway [24,25]. Our study therefore confirmed the role of miR-21 in glioma cell proliferation, and the effect on the glioma was involved in the regulating the PI3K/AKT pathway.

In summary, the present study revealed that miR-21 plays a vital role in brain glioma, and the effects of miR-21 on brain glioma are associated with the regulation of PI3K/AKT pathway. Further studies should be conducted to determine PTEN expression in U251 cells after miR-21 silencing and the relationship between PTEN expression and other cell regulatory factors, in order to provide more evidence for clinical studies. The miR-21/ PI3K/AKT pathway may represent an attractive pharmacological target for the treatment of brain glioma.

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