# Review Article
# Expression of miR-218 in brain glioma and its relationship with AKT/mTOR signal pathway

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**Abstract:** Brain glioma is one common and severe intracranial malignant tumor. Current treatment strategy cannot obtain satisfactory efficacy. Further study of pathogenesis mechanism is thus of critical importance for novel treatment measure. MicroRNA (miR)-218 is closely related with pathogenesis and progression of brain glioma, detailed mechanism of which, however, remains unknown. This study thus investigated the role of miR-218 in brain glioma by examining Akt/mTOR signal pathway. Tumor tissue and adjacent tissue samples were collected from glioma patients. Meanwhile miR-218 mimic or NC plasmid was used to transfect U251 cells. Real-time fluorescent quantitative PCR was employed to detect miR-218 level in both tumor tissue and culture cells. Protein level of p-Akt and p-mTOR was measured by Western blotting. MiR-218 expression level was significantly lower in brain glioma tissues compared to adjacent tissues (t=8.32, P<0.001). MiR-218 level was gradually decreased with higher pathology grade of glioma, which also had remarkably higher level of p-Akt and p-mTOR than normal tissues. The expression level of p-Akt and p-mTOR protein was positively correlated with pathology grade of glioma and was negatively correlated with miR-218 level (r=-0.91 or -0.90, P<0.001). Cultured cells had significantly lower levels of p-Akt and p-mTOR proteins after over-expressing miR-218 (P<0.05). MiR-218 and abnormal expression of Akt/mTOR signal pathways are related with occurrence and prognosis of brain glioma. Mir-218 might exert anti-tumor effects via regulating Akt/mTOR signal pathways.

**Keywords:** Brain glioma, miR-218, Akt, mTOR

## Introduction

Intracranial tumor occupies about 90% of all tumor of central nervous system [1]. Brain glioma is the most common brain tumor, as it contains about 60%~70% of all primary intracranial tumors. Due to the specific position of tumor, brain glioma has high morbidity and mortality. Meanwhile, due to its high invasiveness and proliferation potency [2], the boundary between tumor and normal brain tissue is hard to discriminate, resulting the difficulty in complete remove of tumors in surgery as well as high recurrence rate [3]. Current treatment method of brain glioma mainly consists of surgery, plus radio- and chemo-therapy as complement. However, due to the presence of brain-blood-barrier (BBB), high concentration of chemotherapy agents is unable to present in focal brain regions, largely impeding the efficacy. Therefore, further investigation of the pathogenesis of brain glioma, is of critical importance for gaining more knowledge about tumors and development for novel treatment strategy.

MiroRNA (miRNA) is one group of small noncoding RNA molecules participating in body development and disease occurrence. It has 19~24nt length in eukaryotes, and can bind to 3'UTR of target gene via complete or incomplete complementary binding, to inhibit or degrade target mRNA. MiRNA thus participates the gene expression and progression of various diseases including tumor, cardio-cerebro-vascular disease and immune disorder. Meanwhile, its highly conserved sequence makes miRNA as a potential biomarker. Various studies showed the close correlation between abnormal expression of miRNA and occurrence/progression of brain glioma. Some scholars even suggested that serum miRNA can work as the biomarker for diagnosis, treatment and prognosis predic-

tion of brain glioma [4]. For example, miR-126 was significantly down-regulated in brain glioma tissues, and was closely correlated with tumor's pathology grade [5]. MiR-506 could inhibit proliferation, invasion and metastasis of glioma cells via regulating STAT3 expression [6]. Tang et al reported that serum miR-185 could work as an important marker for predicting the prognosis of brain glioma patients [7].

Recent studies showed the correlation between miR-218 and occurrence/progression of various tumors including gastric carcinoma [8], pulmonary cancer [9, 10], cervical cancer [11, 12] and colon cancer [13] via different target genes. MiR-218 has also been found to inhibit the proliferation of glioma cells [14]. Propofol can inhibit proliferation and invasion of glioma cells via up-regulating miR-218 expression [15], indicating that miR-218 could work as one potential tumor suppressor [16-18] during the occurrence/progression and treatment of brain glioma. Therefore further study regarding the functional mechanism of miR-218 in the occurrence of brain glioma has important implication for its application in targeted treatment. Some study found that miR-218 could inhibit Akt phosphorylation in oral carcinoma cells [19]. In colon cancer, miR-218 was also found to suppress PIK3/Akt/mTOR signal pathway [13]. This study thus investigated the expression level of miR-218 and Akt/mTOR in glioma cells, in an attempt to elucidate their correlations. *In vitro* study was also performed to establish their correlation for revealing the role and mechanism of miR-218 in brain glioma.

**Materials and methods**

*Reagent and equipment*

U251 cell line was purchased from ATCC (Manassas, VA US); Gibco RPMI culture medium, 0.25% trypsin and fetal bovine serum were from Thermo Fisher Scientific (Waltham, MA USA); Trizol RNA extraction reagent and Lipofecatmine 2000 were obtained from Thermo Fisher Scientific (Waltham, MA USA); Reverse transcription kit and real-time fluorescent RT-PCR kit were bought from TaKaRa (TaKaRa, Kusatsu, Shiga, Japan). Gel imaging system and ViiA7 fluorescent quantitative PCR cycler were from ABI (Thermo Fisher Scientific, Waltham, MA USA). Total protein extraction kit and coomassie brilliant blue protein quantification kit were collected from BestBio (Shanghai,

China). SDS-PAGE, PBST buffer, electrophoresis apparatus and GIS-2020D gel imaging system were purchased from Sigma (Merck, Temecula, CA, USA); Antibody of p-Akt, p-mTOR and GAPDH were from Abcam (Cambridge, MA, USA); MiR-218 mimics and miR-218 mimics NC were synthesized from Invitrogen (Thermo Fisher Scientific, Waltham, MA USA); Primers were synthesized by TaKaRa (Kusatsu, Shiga, Japan).

*Research subject*

A total of 41 brain glioma patients from January 2012 to January 2015 in our hospital were recruited, with no age, sex, ethnic, or tumor-stage restrictions. All patients had confirmed diagnosis of brain glioma according to pathology diagnostic criteria stipulated by CNS tumor classification system (WHO, 2007) [20]. There were 23 males and 18 females in total, aging between 25 and 68 years (average =45.2± 8.56). The patients with untreated glioma received histologic spanning, ranging from low-grade, anaplastic to high-grade (GBM). Included in this analysis were 12 GBMs, 9 anaplastic gliomas, and 20 low-grade gliomas. Anaplastic tumors included: 3 anaplastic ependymoma, 4 anaplastic astrocytomas, 1 anaplastic oligodendrogliomas, and 1 anaplastic oligoastrocytoma. Low-grade tumors included: 3 ependymoma, 1 gangliomas, 7 oligodendrogliomas, 2 astrocytomas, 2 pilocytic astrocytoma and 6 subependymoma. All patients gave consents before surgery. Both tumor and adjacent tissues were kept at -80°C for further experiments. Inclusive criteria: (1) No radio- or chemo-therapy before the surgery; (2) Primary tumor; (3) Complete medical history.

*Cell culture and transfection*

U251 cells were cultured in 1640 medium containing 10 FBS in a 37°C chamber with 5% $CO_2$. Cells were passed when reaching 80%~90% confluence. Cells were washed in PBS and digested in trypsin. After preparing cell suspension, 200 ml was employed for culture. U251 cells were then transfected with miR-218 or miR-218 NC control by Lipofectamine 2000 following manual instruction. In brief, 5 μl Lipofectamine 2000 was diluted in 250 μl DMEM, which was then added with 250 μl DMEM containing 7.5 μl miR-218 mimics or NC. The mixture was incubated at room temperature for 20 min to form miR-218 mimics/NC-
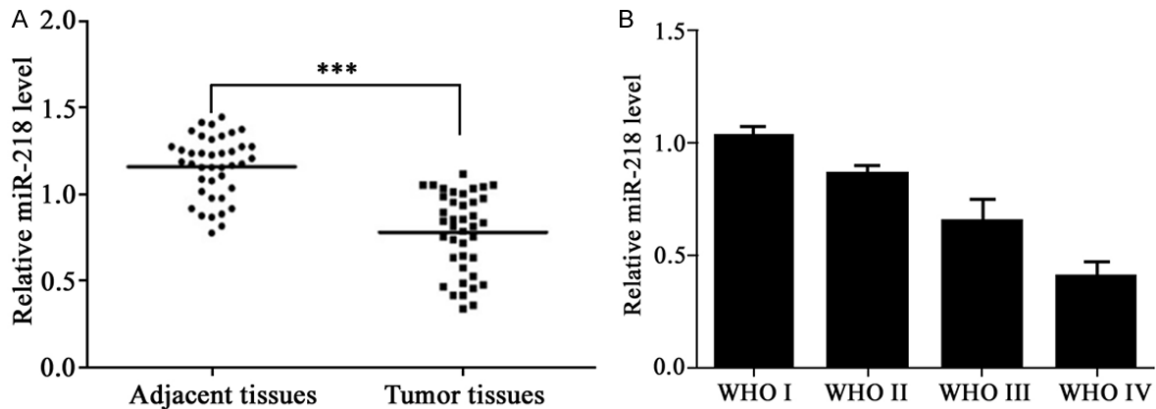
**Figure 1.** Correlation between miR-218 expression in brain glioma and pathological grade. ***P<0.001 between two groups.

Lipofectamine 2000 complex, which was then added into culture medium containing cells. After 24 h incubation, the transfection efficiency was observed under the microscope. Those cells with higher than 80% transfection efficiency were enrolled for further study. MiR-218 and miR-Ctrl groups were assigned using miR-218 mimics or miR-218 NC, respectively, along with normal U251 cells in control group. Cells were continuously cultured for 24 h in following studies.

*Real-time fluorescent quantitative PCR for measuring miR-218 expression level*

Trizol reagent was used to extract RNA from both tissue samples and cultured cells. RNA concentration and purity were determined by automatic nucleic acid analyzer. The integrity of RNA molecules was identified by 1% agarose gel electrophoresis. cDNA was synthesized using *in vitro* reverse transcription from 1 μg RNA following manual instruction. The system of real-time fluorescent quantitative PCR consists of 4.5 μ 2XSYBR Green mixture, 1 μ cDNA, 1 μ of each primer (5 μM), and 2.5 μl ddH$_2$O. The reaction conditions were: 95°C for 30 s, followed by 40 cycles each containing 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The reaction was performed on ViiA7 fluorescent quantitative PCR cycler in triplicates (N=3) using U6 as the internal reference gene.

*Western blotting for protein levels of p-Akt and p-mTOR*

Total proteins were extracted from both cells and tumor tissues using protein extraction kit following manual instruction. After centrifuga-

tion, coomassie brilliant blue reagent was used to quantify proteins, which were boiled for 5 min. 8% and 16% SDS-PAGE separation gel buffers were prepared to generate a gradient separation gel, which was blocked by absolute ethanol. After settle down, the gel comb was removed. 10 ml 4% SDS-PAGE stacking gel was then prepared for loading proteins into each well. Electrophoresis was performed at 12 mA constant current in stacking gel, and was switched to 9 mA in the phase of separation gel for 8 h. After electrophoresis, the stacking gel was placed on the cathode while NC membrane was placed on the anode. The transfer sandwich was placed into the chamber of electrical transfer apparatus, with the addition of transfer buffer. Under 300 mA constant current for 1.5 h, the membrane was then blocked in PBST containing 5% defatted milk powder for 2 h at room temperature. Primary antibody dilutions were then added following PBST washing (3 times) for 4°C overnight incubation. Secondary antibody in PBST with 2.5% defatted milk powder was then added for 60-min room temperature incubation. After rinsing the membrane for three times, chromogenic substrates were applied evenly on NC membrane, which was exposed in a dark room. The film was placed in developing buffer for several minutes. After washing out developing buffer, the film was fixed in fixing buffer and was dried. GIS-2020D gel imaging system was used to analyze optical density (OD) values of p-Akt, p-mTOR and GAPDH bands. Using the ratio against OD values of GAPDH band, the relative expression intensity of p-Akt and p-mTOR proteins was calculated.

**Table 1.** Relative expression intensity of p-Akt and p-mTOR proteins

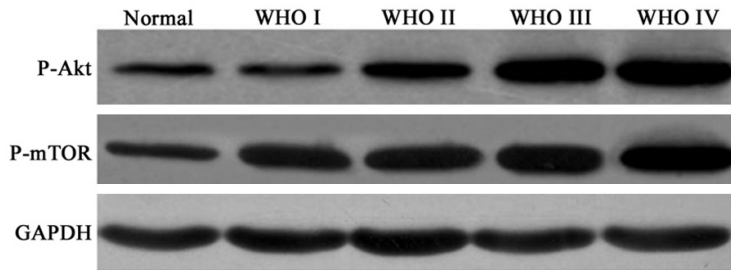|  | Normal tissue | WHO I | WHO II | WHO III | WHO IV |
|---|---|---|---|---|---|
| p-Akt | 0.56±0.12 | 0.67±0.08 | 0.86±0.07 | 1.00±0.08 | 1.13±0.06 |
| p-mTOR | 0.61±0.13 | 0.72±0.07 | 0.90±0.11 | 1.08±0.07 | 1.17±0.09 |



**Figure 2.** Expression of p-Akt and p-mTOR in brain glioma tissues.

*Statistical analysis*

SPSS13.0 software was used for statistical analysis. Those samples fitted normal distribution were compared by two-sample independent t-test. Pearson correlation analysis was performed for two-variable analysis. Those data did not fitted normal distribution was analyzed by Spearman correlation analysis. A significant level was defined when P<0.05.

**Results**

*Expression of miR-218 in brain glioma and its correlation with pathological grade*

Using real-time fluorescent quantitative PCR, we measured the expression of miR-218 in tumor and adjacent tissues of all 41 brain glioma patients. As shown in **Figure 1A**, the expression level of miR-218 in tumor tissues was significantly lower than that of adjacent tissues (0.77±0.24 vs. 1.16±0.18, t=8.32, P<0.001).

Based on WHO classification system, there were 13, 10, 10 and 8 cases of grades I, II, III, and IV of brain glioma samples. As shown in **Figure 1B**, miR-218 expression level was gradually decreased with higher pathology grade of brain glioma.

*Relationship between p-Akt and p-mTOR proteins and pathological grade of brain glioma*

Western blotting was employed to check the expression of p-Akt and p-mTOR in tumor and adjacent tissues from all 41 brain glioma pa-tients. As shown in **Table 1** and **Figure 2**, p-Akt level was significantly higher in glioma tissues compared to normal tissues (0.89±0.19 vs. 0.56±0.12, t=9.40, P<0.001). Similar patterns were revealed for p-mTOR (0.94±0.19 vs. 0.61± 0.13 for tumor and normal tissues, respectively; t=9.18, P< 0.001). Spearman correlation analysis was used to detect the relationship between pathological grade and expression intensity of p-Akt and p-mTOR proteins. Results showed significantly positive correlation between those parameters (r= 0.89 for p-Akt, r=0.90 for p-mTOR, P<0.001 in both cases). With advanced pathological grade of glioma, expression levels of both p-Akt and p-mTOR were elevated.

*Correlation between p-Akt and p-mTOR proteins and miR-218 level*

Using Pearson correlation analysis, the relationship between protein expression levels of p-Akt and p-mTOR and miR-218 level was shown in **Figure 3**. A significantly negative relationship existed between those two proteins and miR-218 level (r=-0.91 for p-Akt and -0.90 for p-mTOR, P<0.001 in both cases).

*MiR-218 expression in U251 cells after transfection*

To substantiate the effect of miR-218 mimics transfection on U251 cells, real-time fluorescent quantitative PCR was employed to test miR-218 levels in all cells. As shown in **Figure 4**, miR-218 mimics-transfected cells had significantly higher miR-218 relative level compared to either of miR-218 NC transfected cells or un-transfected control cells (6.47±0.15 vs. 1.00±0.17 or 1.03±0.13, P<0.01 in both cases). No significant difference was found regarding miR-218 expression level between miR-Ctrl and control group (P>0.05). Results showed elevated miR-218 expression level in U251 cells after miR-218 mimics transfection, thus paving grounds for further experiments.
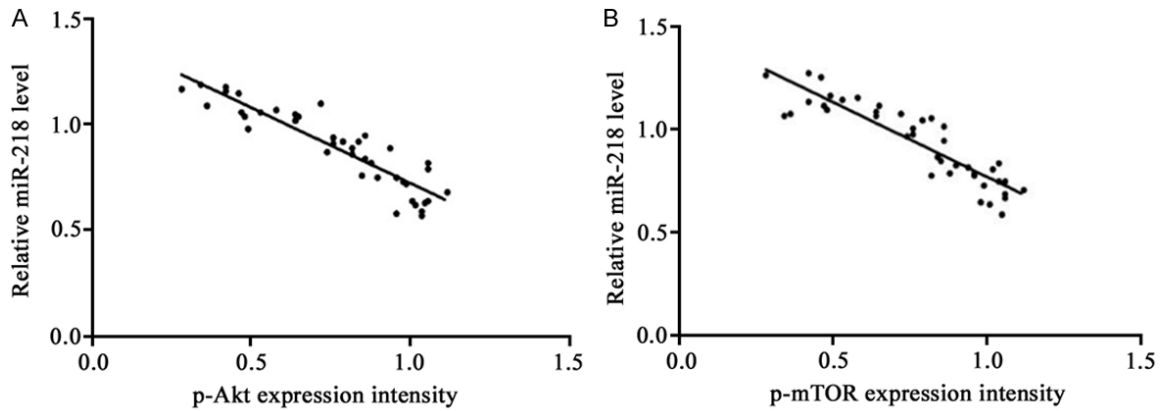
**Figure 3.** Correlation between p-Akt (A) or p-mTOR (B) protein and miR-218 expression level.



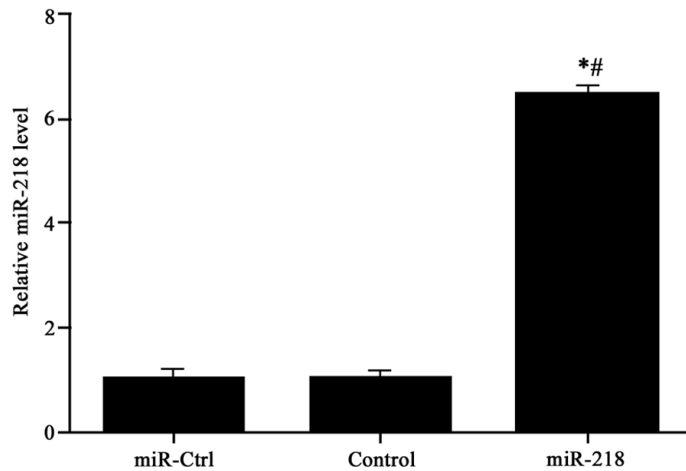**Figure 4.** MiR-218 expression in U251 cells after miR-218 mimics transfection. *, p<0.01 compared to miR-Ctrl group; #, p<0.01 compared to control group.
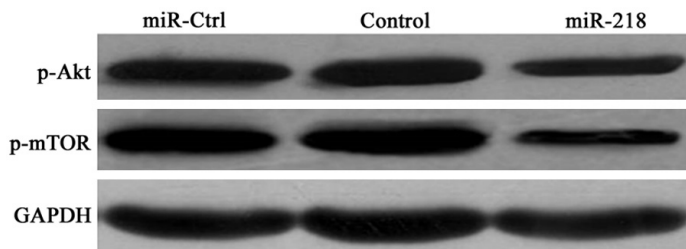


**Figure 5.** Protein expression levels of p-Akt and p-mTOR in U251 cells after transfection.

*Protein expression level of p-Akt and p-mTOR in U251 cells after transfection*

After transfecting U251 cells with miR-218 mimics or NC, western blotting was employed to quantify protein levels of p-Akt and p-mTOR in all groups of cells. As shown in **Figure 5**, p-Akt protein level in miR-218 was significantly lower than that of miR-Ctrl or control group (0.53±0.08 vs. 1.04±0.09 or 1.05±0.10 for miR-Ctrl or control groups, respectively; P<0.05 in both cases). No significant difference existed between miR-Ctrl and control group (P>0.05). Similar patterns also occurred for p-mTOR proteins, as miR-218 transfected cells had significantly lower expression level compared to miR-Ctrl or control group (0.49±0.07 vs. 1.02±0.11 or 1.04±0.12 for miR-Ctrl or control group, respectively; P<0.05 for both cases). No significant difference existed between miR-Ctrl and control group (P>0.05).

## Discussion

Tumor occurrence and progression is the result of multiple factors, including inactivation of tumor suppressor gene and activation of oncogene. It is widely accepted that miRNA exerts similar functions as those in tumor suppressor gene or oncogene. Previous study has established the suppression of tumor progression by miR-218 in various cancers including brain glioma [21, 22]. MiR-218 expression was also known to be closely related with the prognosis of brain glioma [23]. Therefore the study of the functional mechanism of miR-218 during pathogenesis of brain glioma is of critical importance for clinical treat-

ment. Previous studies found the important role Akt/mTOR signal pathway for both pathogenesis and prognosis of brain glioma [24, 25]. The role of miR-218 in Akt/mTOR signal pathway has also been revealed in other tumors. Therefore the study of the correlation between miR-218 and Akt/mTOR signal pathway was studied here.

By examining the expression profile of miR-218 and Akt/mTOR signal pathways in both brain glioma and normal tissues, it was found that miR-218 was significantly down-regulated in brain glioma tissues, with negative correlation between miR-218 level and pathology grade, suggesting the role of miR-218 in the occurrence of brain glioma and its potency as biomarker for pathology grade. This was consistent with Cheng et al [23], and paved grounds for the role of miR-218 as biomarker for brain glioma. Moreover, the expression levels of p-Akt and p-mTOR were significantly elevated in brain glioma, with positive correlation with the malignancy of tumors, suggesting the involvement of Akt/mTOR signal pathway alternation in the pathogenesis of brain glioma. The protein level of Akt/mTOR pathway could also work as biomarker reflecting tumor pathology grade. Li et al found the participation of p-Akt, p-mTOR and p-p70S6K in the malignancy of brain glioma [26], as consistent with our results. The analysis between miR-218 expression level and protein expression of p-Akt/p-mTOR found significantly negative correlation between miR-218 and those two proteins. In cells with over-expression of miR-218, the protein level of p-Akt and p-mTOR was significantly lowered, suggesting the regulation of Akt/mTOR signal pathway by miR-218 in brain glioma. Similar results have been discovered in other tumors. For example, Li et al found that miR-218 could inhibit p-Akt expression in cervical cancer [27], and Zhang et al found that invasion and metastasis of colon carcinoma can be inhibited by miR-218 via mediating PI3K/Akt/mTOR signal pathways [13].

This study for the first time demonstrated the regulation of miR-218 on Akt/mTOR signal pathway. We initially discovered the correlation between miR-218 and Akt/mTOR, whose expression levels were then detected in cells over-expressing miR-218. Our study illustrated the regulation of miR-218 on Akt/mTOR signal pathway, providing further evidences for under-

standing the functional mechanism of miR-218 in brain glioma, and paving the ground for its application in targeted treatment in clinics. Meanwhile, new insights have been revealed regarding the diagnosis, treatment and prognosis of brain glioma, using miR-218 as one potential biomarker.

## Disclosure of conflict of interest

None.

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