

# Growth-inhibitory and chemosensitizing effects of microRNA-31 in human glioblastoma multiforme cells

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**Abstract.** The constitutive activation of signal transducer and activator of transcription 3 (STAT3) contributes to resistance to temozolomide (TMZ) in glioblastoma multiforme (GBM). The aim of this study was to explore the biological role of microRNA-31 (miR-31) in GBM, particularly its role in the regulation of TMZ chemosensitivity. For this purpose, the human GBM cell lines, U251 and U87, were transfected with a miR-31 precursor (pre-miR-31), and cell proliferation, apoptosis and STAT3 phosphorylation were then assessed. To evaluate the effects of miR-31 on TMZ cytotoxicity, the cells were transfected with pre-miR-31 and exposed to 100  $\mu$ M TMZ for 72 h prior to cell proliferation and apoptosis analysis. A constitutively active STAT3 mutant was co-transfected with pre-miR-31 into the cells to confirm the mediating role of STAT3 signaling. The enforced expression of miR-31 significantly reduced cell proliferation and induced mitochondrial apoptosis, as manifested by the loss of mitochondrial membrane potential and the increase in caspase-9 and caspase-3 activity. The phosphorylation level of STAT3 was significantly decreased by the overexpression of miR-31. The co-delivery of the constitutively active STAT3 mutant blocked the tumor suppressive effects of miR-31. In addition, miR-31 overexpression significantly enhanced the cytotoxic effects of TMZ on the GBM cells, as evidenced by the accelerated suppression of cell proliferation and the induction of apoptosis. The chemosensitizing effects of miR-31 were significantly impaired by the expression of the constitutively active STAT3 mutant. Taken together, our results indicate that miR-31 triggers mitochondrial apoptosis and potentiates TMZ cytotoxicity in GBM cells largely through the suppression of

STAT3 activation. Thus, the restoration of miR-31 expression may be of therapeutic benefit in the treatment of GBM.

## Introduction

Glioblastoma multiforme (GBM) is the most common and lethal primary malignancy of the central nervous system, and patients with GBM have a poor prognosis (1). Current standard therapies for GBM comprise surgical resection, chemotherapy and radiotherapy (2). Temozolomide (TMZ), a DNA methylating agent, is the primary chemotherapeutic drug used in the treatment of malignant gliomas (3). Clinical studies have indicated that TMZ chemotherapy alone or in combination with radiotherapy increases the survival rate of patients with GBM (4,5). Despite advances in treatment strategies, the median survival rate of patients with GBM is only 12-14 months (6).

Chemoresistance is a major obstacle to effective cancer chemotherapy. TMZ induces the formation of O<sup>6</sup>-methylguanine in DNA, which consequently leads to DNA replication defects and cell death. A well-established mechanism for resistance to TMZ is mediated by the DNA repair protein O<sup>6</sup>-methylguanine methyltransferase (MGMT), which repairs TMZ-induced DNA lesions through the removal of the O<sup>6</sup>-methyl group (7). Additionally, the aberrant activation of survival signaling pathways also contributes to the resistance of GBM cells to TMZ (8). Signal transducer and activator of transcription 3 (STAT3) signaling has been implicated in the development and progression of many types of tumor, including GBM (9). The constitutive activation of STAT3 has been shown to contribute to the resistance of GBM cells to TMZ (10), and thus STAT3 represents an important therapeutic target for this disease.

MicroRNAs (miRs or miRNAs) are a class of endogenous, non-coding regulatory RNAs of ~22 nucleotides in length. They regulate a large number of target genes and thus affect various biological processes, such as cell proliferation, differentiation, apoptosis, invasion and tumorigenesis (11,12). miR-31 is dysregulated in many human malignancies, such as bladder cancer (13), melanoma (14) and colorectal cancer (15). Compared to normal brain tissue, miR-31 expression is significantly decreased in glioma tissue (16). miR-31 functions as an oncogene or a tumor suppressor in different types of cancer (14,15,17). For instance, miR-31 has been shown to enhance the proliferation of colon cancer cells (17), whereas in melanoma cells, miR-31 has been shown to inhibit cell migra-

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tion and invasion (14). Despite these studies, relatively little is known about the biological role of miR-31 in GBM, particularly in relation to the regulation of chemosensitivity.

In this study, we investigated the effects of miR-31 overexpression on the proliferation, apoptosis and TMZ sensitivity of GBM cells, and we also examined the involvement of STAT3 signaling.

## Materials and methods

**Cell culture.** Normal human astrocytes were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and the human GBM cell lines, U251 and U87, were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 25 mmol/l D-glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and penicillin (100 U/ml)-streptomycin (100 µg/ml; all from Invitrogen).

**Measurement of miR-31 expression.** miR-31 expression was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), as previously described (18). In brief, total RNA was extracted from the cells using TRIzol reagent (Invitrogen), and cDNA was synthesized with specific stem-loop reverse transcription primers. miR-31 expression levels were measured using TaqMan microRNA assays (Taqman<sup>®</sup> MicroRNA Reverse Transcription kit; Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 95°C for 5 min, followed by 40 cycles of amplification (95°C for 20 sec, 60°C for 20 sec and 72°C for 30 sec). The relative level of miR-31 following normalization to U6 small nuclear RNA was analyzed using the comparative cycle threshold ( $\Delta\Delta C_t$ ) method, as previously described (19).

**Plasmids, miRNA oligonucleotides and cell transfection.** The STAT3-C plasmid expressing the constitutively active STAT3 mutant was purchased from Addgene (Cambridge, MA, USA), as previously described (20). The pcDNA 3.1(+) control vector was purchased from Invitrogen. Human pre-miR-31 and pre-miR negative control oligonucleotides were purchased from GenePharma Corp. (Shanghai, China). Cell transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Untransfected cells were used as the controls. The final concentration of each miRNA oligonucleotide was 50 nM. To determine the mediating role of STAT3, 1 µg of STAT3-C or the control plasmid was co-transfected into the GBM cells. At 24 h post-transfection, the cells were subjected to cell proliferation, apoptosis and gene expression analyses.

**Treatment with TMZ.** To determine the effects of miR-31 on TMZ cytotoxicity, the cells were transfected with pre-miR-31 or co-transfected with pre-miR-31 and STAT3-C 24 h prior to exposure to 100 µM TMZ, as previously described (21). Following incubation for an additional 72 h, the cells were harvested for cell proliferation and apoptosis assays.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** The cells were seeded in 96-well microplates at a density of  $4 \times 10^3$  cells/well. Following treatment, the cells

were subjected to viability analysis using an MTT assay. MTT solution (5 mg/ml; Sigma, St. Louis, MO, USA) was added to each well followed by incubation at 37°C for 4 h. Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a spectrophotometer (DU-640; Beckman Coulter, Hialeah, FL, USA).

**Apoptosis assay.** Following treatment, the cells were harvested by trypsinization, and apoptosis was detected using the Annexin V apoptosis kit (Becton-Dickinson Biosciences, San Diego, CA, USA). The cells were stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI) solution (20 µg/ml) for 15 min in the dark. Apoptotic cells (Annexin V-positive) were analyzed by flow cytometry (Becton-Dickinson Biosciences).

**Measurement of mitochondrial membrane potential ( $\Delta\Psi_m$ ).** Changes in the  $\Delta\Psi_m$  were measured using the JC-1 mitochondrial membrane potential assay kit (Beyotime, Nantong, China). JC-1 forms monomers that emit green fluorescence and JC-1 aggregates are marked by red fluorescence. The ratio of JC-1 green to red fluorescence is proportional to the strength of  $\Delta\Psi_m$ . At 72 h post-transfection, cells were collected by trypsinization and pelleted. The cells were resuspended in JC-1 working solution and incubated at 37°C for 15 min. After washing, the stained cells were analyzed by flow cytometry (Becton-Dickinson Biosciences).

**Measurement of caspase-3 and caspase-9 activity.** The activity of caspase-3 and caspase-9 was measured using the Caspase-3/9 Activity assay kit (Beyotime), according to the manufacturer's instructions. Briefly, the transfected cells were lysed and the lysates were incubated with reaction buffer, which contained the fluorescent substrates, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) and N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA), for caspase-3 and caspase-9, respectively. The fluorescence of the cleaved substrates was determined using an SLM 8000 fluorometer (SLM-Aminco, Urbana, IL, USA) at 405 nm.

**Western blot analysis.** For western blot analysis, the following primary antibodies were used: rabbit anti-total STAT3 (sc-482), anti-phosphorylated (p-)STAT3 (sc-8001-R), anti-cyclin D1 (sc-753), anti-Mcl-1 (sc-819) and anti-survivin (sc-10811) polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti- $\beta$ -actin (A5316) monoclonal antibody (Sigma). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (#31460) or anti-mouse (#31430) IgGs were purchased from Pierce Biotechnology (Rockford, IL, USA). Following treatment, the cells were lysed in RIPA buffer (Sigma) containing 1 mM phenylmethanesulfonyl fluoride and complete protease inhibitors (Roche, Mannheim Germany). The protein concentrations in cellular lysates were measured using a BCA Protein assay kit (Thermo Scientific, Rockford, IL, USA). The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% fat-free milk and incubated with the primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies for 1 h. Immunoreactive

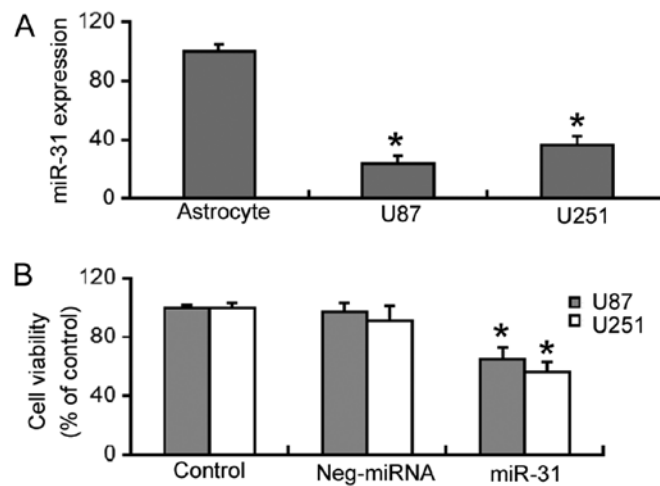


Figure 1. Effect of miR-31 overexpression on the viability of glioblastoma multiforme (GBM) cells. (A) RT-qPCR was performed to measure the miR-31 expression levels in U87 and U251 GBM cells and normal human astrocytes. \* $P < 0.05$  vs. astrocytes. (B) Cell viability was detected by MTT assay. Cells were transfected with miR-31 precursor or negative miRNA (Neg-miRNA) and subjected to MTT assay. Bars represent the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  vs. cells transfected with negative miRNA. Control, untransfected cells.

bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) and quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Statistical analysis.** Data are expressed as the means  $\pm$  standard deviation (SD). Differences between groups were examined by one-way analysis of variance using the SPSS software package v19.0 (SPSS, Inc., Chicago, IL, USA). A  $P$ -value  $< 0.05$  was considered to indicate a statistically significant difference.

## Results

**Overexpression of miR-31 inhibits the viability of GBM cells.** RT-qPCR revealed that miR-31 expression was significantly decreased ( $P < 0.05$ ) in the U87 and U251 cells, compared to normal human astrocytes (Fig. 1A). The effects of restoring miR-31 expression on the viability of GBM cells was then investigated. As shown in Fig. 1B, miR-31-overexpressing U87 and U251 cells had a significantly lower viability ( $P < 0.05$ ) compared to the control (untransfected) or negative miRNA-transfected cells.

**Overexpression of miR-31 significantly increases the apoptosis of GBM cells through the mitochondrial pathway.** Subsequently, we examined the effects of miR-31 overexpression on the survival of GBM cells. Apoptosis detection by Annexin V/PI staining demonstrated that the enforced expression of miR-31 induced a significant increase ( $P < 0.05$ ) in the apoptosis of the U87 and U251 cells (Fig. 2A). As the depolarization of  $\Delta\Psi_m$  is an early event in mitochondrial-related apoptosis, we measured the changes in  $\Delta\Psi_m$  induced by miR-31 overexpression using the JC-1 dye. As shown in Fig. 2B, miR-31 overexpression resulted in a significant decrease ( $P < 0.05$ ) in the red/green fluorescence ratio, which was indicative of the loss of  $\Delta\Psi_m$ . Enzyme activity assay revealed that there was a 2.4- and 2.8-fold increase in caspase-9 activity and an 8.6- and 12.4-fold increase in caspase-3 activity in miR-31-overexpressing U87 and U251 cells, respectively (Fig. 2C and D).

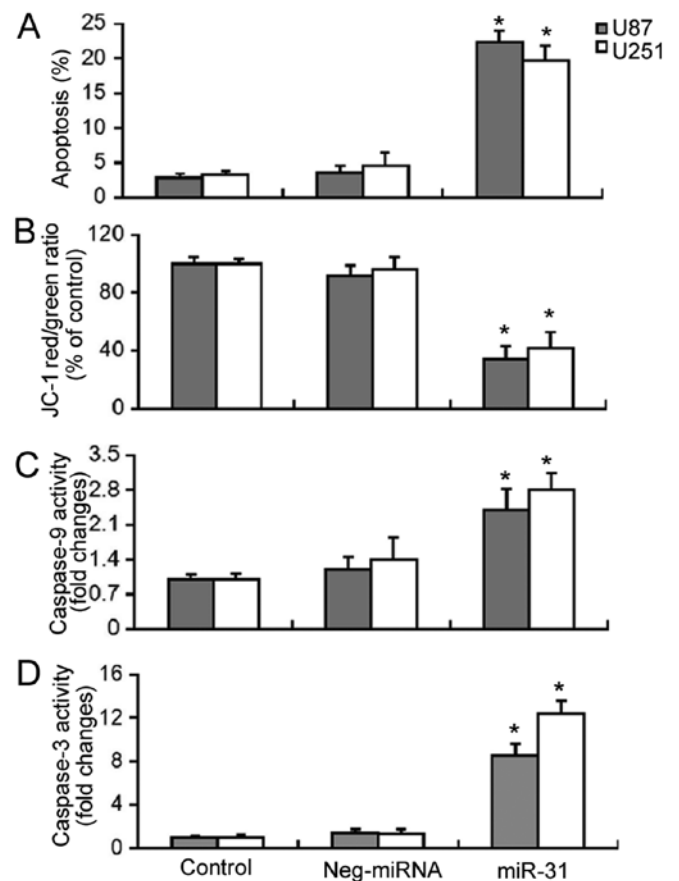


Figure 2. Overexpression of miR-31 induces mitochondrial apoptosis in glioblastoma multiforme (GBM) cells. Cells were left untreated (control) or were transfected with miR-31 precursor or negative miRNA (Neg-miRNA). Subsequently, cells were tested for apoptotic changes. (A) Cells stained with Annexin V and propidium iodide (PI) were analyzed by flow cytometry. Annexin V-positive cells were considered apoptotic. (B) Alterations in mitochondrial membrane potential ( $\Delta\Psi_m$ ) were measured by flow cytometry using JC-1 staining, as described in the Materials and methods. The results are expressed as percentages of the control values (assigned 100%). The activity of (C) caspase-9 and (D) caspase-3 was measured by fluorogenic assay. Bar graphs represent the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  vs. cells transfected with Neg-miRNA.

*Restoration of miR-31 interferes with the activation of STAT3 signaling in GBM cells.* It is known that the constitutive activation of STAT3 signaling contributes to GBM growth and survival (9). Thus, we examined the effects of restoring miR-31 expression on the activation of STAT3 signaling. As shown in Fig. 3A, in the miR-31-overexpressing U87 and U251 cells, a significant decrease ( $P < 0.05$ ) in the phosphorylation of STAT3 was noted, compared to the controls and negative miRNA-transfected cells. We also examined the effects of restoring miR-31 expression on the expression of target genes of STAT3, including cyclin D1, Mcl-1 and survivin. Western blot analysis demonstrated that miR-31 overexpression markedly inhibited the expression of cyclin D1, Mcl-1 and survivin in the U87 and U251 cells (Fig. 3B).

*miR-31 enhances the efficacy of TMZ in GBM cells.* We then investigated the effects of miR-31 overexpression on the sensitivity of GBM cells to TMZ. MTT assay revealed that TMZ at 100  $\mu\text{M}$  induced  $\sim 20\%$  decrease in the viability of the U87 and U251 cells after a 72-h incubation (Fig. 4A). Notably, the delivery of miR-31 significantly enhanced ( $P < 0.05$ ) the cytotoxic effects of TMZ on GBM cells, leading to a 60-70% decrease in cell viability (Fig. 4A). Similarly, the overexpression of miR-31 significantly increased ( $P < 0.05$ ) the apoptosis of the TMZ-treated GBM cells (Fig. 4B).

*Chemosensitizing effect of miR-31 is mediated through the inactivation of STAT3.* Having concluded that STAT3 signaling was suppressed by miR-31 overexpression, we examined the involvement of STAT3 signaling in miR-31-induced chemosensitization. Co-transfection with a constitutively active STAT3 mutant markedly enhanced the phosphorylation of STAT3 in the miR-31-transfected GBM cells (Fig. 5A). Of note, the constitutive activation of STAT3 significantly reversed ( $P < 0.05$ ) the enhancement of TMZ cytotoxicity which was achieved by miR-31 overexpression (Fig. 5B).

## Discussion

Certain miRs are deregulated in GBM and have been implicated in tumor growth and survival (22,23). Yue *et al* (23) demonstrated that miR-205 was significantly downregulated in human glioblastoma cells and that the restoration of its expression induced apoptosis and cell cycle arrest in glioma cells. Zhang *et al* (24) reported that miR-195 plays a tumor-suppressor role in human glioblastoma cells, impairing cell cycle progression and cellular invasion. Similarly, our data demonstrated that miR-31 functions as a tumor suppressor in GBM, as evidenced by the observation that the ectopic expression of miR-31 significantly suppressed GBM cell proliferation and induced apoptosis. Our data further demonstrated that miR-31 induced the apoptosis of GBM cells through the mitochondrial cascade, which was manifested by the loss of  $\Delta\Psi\text{m}$  and the activation of caspase-3 and caspase-9. The tumor-suppressive effects of miR-31 have also been documented in several other types of cancer, such as melanoma (14) and breast cancer (25). However, there is also evidence that miR-31 plays an oncogenic role in certain human tumors. For instance, Mitamura *et al* (26) reported that miR-31 facilitates the anchorage-independent growth and tumorigenesis of endometrial cancer cells. In non-small cell lung cancer

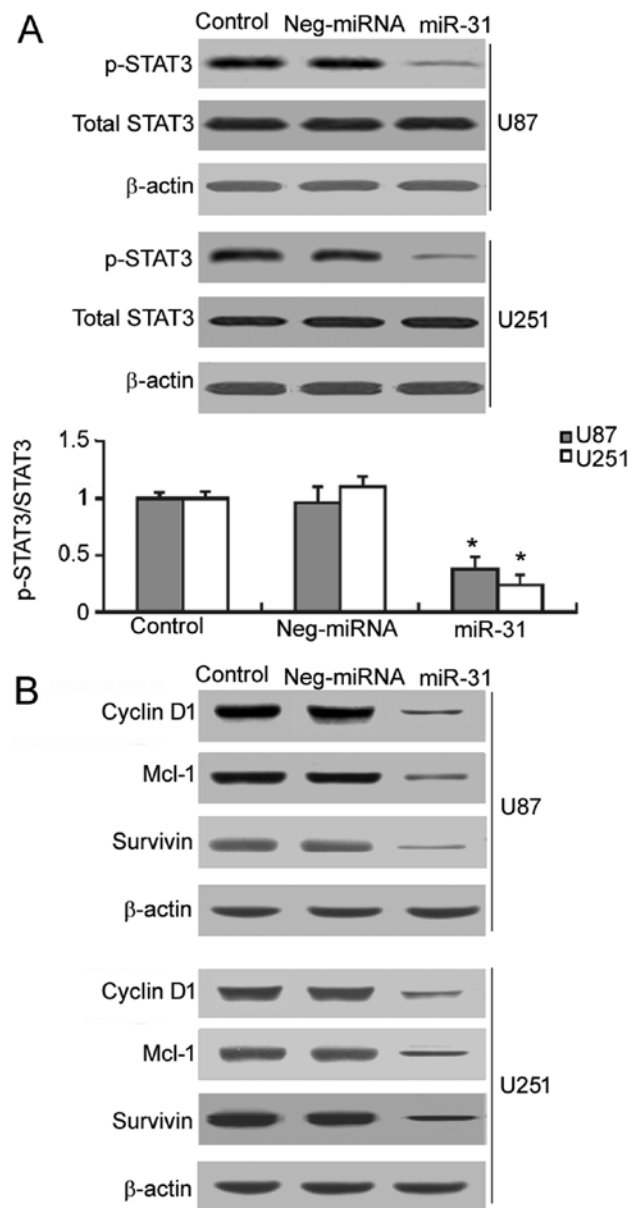


Figure 3. Restoration of miR-31 interferes with the activation of signal transducer and activator of transcription 3 (STAT3) signaling in glioblastoma multiforme (GBM) cells. Cells remained untreated (control) or were transfected with miR-31 precursor or negative miRNA (Neg-miRNA). Western blot analysis was carried out to measure the changes in the expression of the indicated proteins. (A) Suppression of STAT3 phosphorylation by miR-31. Bar graphs represent the means  $\pm$  SD of 3 independent experiments relative to untreated cells (control). \* $P < 0.05$  vs. cells transfected with Neg-miRNA. (B) Downregulation of cyclin D1, Mcl-1 and survivin in miR-31-overexpressing cells. Representative blots of 3 independent experiments are shown.

cells, it was demonstrated that miR-31 has the ability to inhibit cisplatin-induced apoptosis (27). Therefore, these data suggest that miR-31 regulates tumor growth and survival in a cancer type-dependent manner.

The constitutive activation of Stat3/STAT3 plays an important role in the development and progression of GBM (9). It has been reported that the nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ )-induced STAT3 activation contributes to aggressive phenotypes in GBM and that the inhibition of STAT3 signaling retards the growth of human GBM xenografts (28). The pharmacological

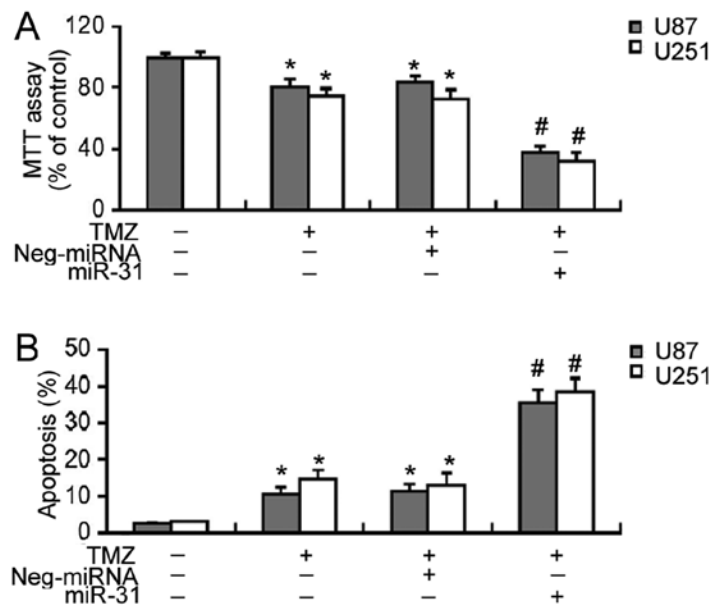


Figure 4. miR-31 enhances the efficacy of temozolomide (TMZ) against glioblastoma multiforme (GBM) cells. Cells were exposed to TMZ (100  $\mu$ M) alone or transfected with negative miRNA (Neg-miRNA) or miR-31 precursor prior to treatment with TMZ. After 72 h of incubation, (A) cell viability and (B) cell apoptosis were assessed. (A) Cell viability was detected by MTT assay. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells transfected with Neg-miRNA.

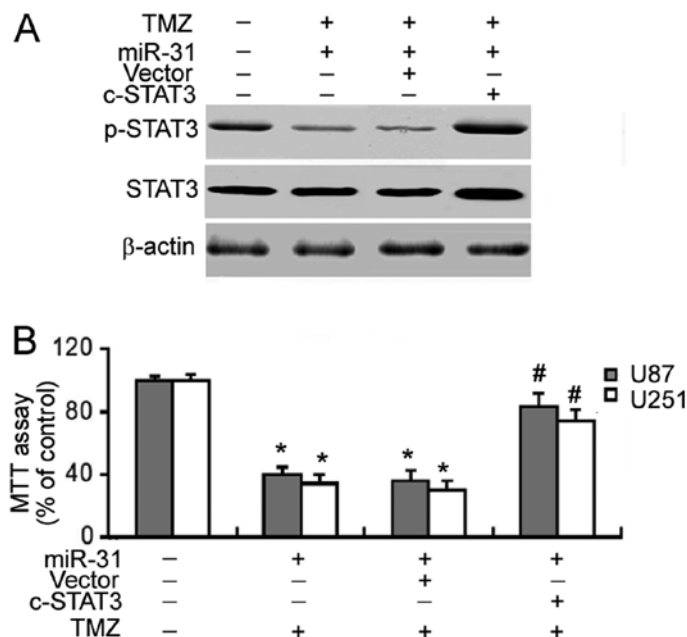


Figure 5. Co-transfection of a constitutively active signal transducer and activator of transcription 3 (STAT3) mutant impairs the chemosensitizing effect of miR-31. Cells were transfected with miR-31 precursor alone or together with empty vector or constitutively active Stat3 mutant (c-STAT3) prior to treatment with temozolomide (TMZ). (A) Western blot analysis of the phosphorylation of STAT3 protein. Representative blots of 3 independent experiments are shown. (B) Cell viability detected by MTT assay. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells co-transfected with miR-31 and empty vector.

inhibition of STAT3 signaling has been found to induce the apoptosis of human GBM cells (29). Of note, our data revealed that the overexpression of miR-31 impaired the activation of STAT3. Moreover, the ectopic expression of miR-31 markedly downregulated multiple STAT3 target genes, including cyclin D1, Mcl-1 and survivin in GBM cells. The downregulation of cyclin D1 is linked to the reduced proliferation and enhanced apoptosis of glioblastoma cells (30). Mcl-1 and survivin are two key pro-survival proteins, and their inhibition contributes to the apoptosis of GBM cells through the

mitochondrial death pathway (31). These studies, combined with our findings, suggest that STAT3 signaling is involved in the tumor-suppressive activity of miR-31 in GBM.

Previous research corroborates the importance of STAT3 signaling in the development of resistance to TMZ in GBM (32). Kohsaka *et al* (10) reported that the inhibition of STAT3 signaling can help overcome resistance to TMZ in glioblastoma. Given our knowledge of the regulation of STAT3 activation by miR-31, in this study, we examined the effects of miR-31 on TMZ chemosensitivity in GBM cells. Notably, we

found that miR-31 overexpression significantly enhanced TMZ cytotoxicity to GBM cells. Moreover, the enforced expression of miR-31 enhanced the apoptosis of GBM cells in the presence of TMZ. The miR-31-mediated chemosensitization to TMZ was reversed by co-transfection with a constitutively active STAT3 mutant. Taken together, these data suggest that the restoration of miR-31 expression sensitizes GBM cells to TMZ largely through the suppression of STAT3 activation. The chemosensitizing activity of miR-31 has also been described in relation to ovarian cancer cells, where the re-introduction of miR31 was shown to re-sensitize paclitaxel-resistant cells to this agent (33).

In conclusion, our data confirm that miR-31 functions as a tumor suppressor gene in GBM. The restoration of miR-31 expression induced the apoptosis of and enhanced TMZ cytotoxicity in human GBM cells, which was mediated through the suppression of STAT3 activation. The re-expression of miR-31 may thus represent a promising strategy for improving the efficacy of TMZ in GBM.

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