# Expression and function of miR-27b in human glioma

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**Abstract.** Our previous miRNAs profiling study showed that miR-27b was up-regulated in glioma cells compared with H4 low grade astrocytoma cells. However, the main function of miR-27b in glioma in not known yet. The aim of this study was to investigate the expression and function of miR-27b in the pathogenesis of glioma. Real-time PCR showed that miR-27b was up-regulated in glioma samples and glioma cells. Down-regulation of miR-27b triggered growth inhibition, induced apoptosis and inhibited invasion in glioma cells. Furthermore, TOPflash luciferase activity was decreased significantly, while FOPflash luciferase did not change significantly. In addition, Western blot assay showed that STAT3, c-myc and cyclin D1 were knocked down after treatment with miR-27b inhibitor. These findings suggest that aberrantly up-regulated miR-27b may be one of the critical factors that contribute to malignancy in human gliomas.

## Introduction

MicroRNAs are located in noncoding regions or the introns of the genome. Mature functional miRNAs of approximately 22 nucleotides generated from long pri-miRNA transcripts control gene expression at the post-transcriptional level by degrading or repressing target mRNAs. MiRNAs are evolutionarily selected gene regulatory molecules. Each type of cell is likely to have a specific miRNA milieu to control gene expression (1). MicroRNAs are predicted to regulate the expression of approximately one-third of all human genes (2,3). Some miRNA can target hundreds of genes, and some genes may be targeted by multiple miRNAs, suggesting that miRNAs play important roles in coordinating many cellular processes (4,5), such as regulating apoptosis, proliferation, differentiation, development, and metabolism.

Previously, some miRNAs aberrantly expressed in cancer have been well documented. They were found to regulate the expression of signaling molecules, such as cytokines, growth factors, transcription factors, and pro-apoptotic and anti-apoptotic genes, and some miRNAs may function as tumor suppressors or oncogenes involved in the pathogenesis of tumors (6). One example of a tumor suppressor miRNA is miR-7, whose confirmed targets include the epidermal growth factor receptor. MiR-7 is frequently found decreased in glioblastoma, while overexpression reduces cell proliferation, survival and invasiveness in cultured glioma cells (7). In contrast, miR-21 is almost invariably overexpressed in glioblastoma and a number of other tumor types, resulting in enhanced cell motility, migration and decreased apoptosis (8-10). Thus, oncogenes and tumor-suppressor genes could be potentially regulated by miRNAs, and miRNAs are presumed to be a class of genes involved in human tumorigenesis.

In our previous studies, we profiled miRNA expression in five glioblastoma cell lines, one astrocytoma cell line, and normal brain tissue. Our data revealed that miR-27b was one of the up-regulated microRNAs (11). To confirm the accuracy of microarray, we use real-time PCR to analyze the expression of miR-27b both in different grade glioma samples and glioma cell lines. We further confirmed that miR-27b was highly expressed in glioma. Using a loss-of-function antisense approach, we demonstrated that down-regulation of miR-27b significantly inhibited proliferation, induced apoptosis and suppressed invasion of glioma. Furthermore, we established that the effect of miR-27b was partly mediated by β-catenin/Tcf-4 activities. These results identify a critical role for miR-27b in regulation of tumor growth in glioma, suggesting that miR-27b acts as an onco-miRNA in gliomagenesis.

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## Materials and methods

*Cell lines and culturing conditions*. Human glioma cell lines, U87, LN229, SNB19, U251 and human astrocyte, were purchased from Chinese Academy of Sciences Cell Bank. All cells were maintained at a 37˚C, in 5% $CO_2$ incubator in DMEM supplemented with 10% fetal bovine serum (FBS), and routinely passaged at 2- to 3-day intervals. Experiments were divided into three groups: the blank control group, miRNA scrambled group, and the As-miR-27b group.

*RNA isolation*. Human glioma tissue samples were obtained from the Huanhu Hospital of Tianjin Medical University after informed consent was obtained from adult patients diagnosed with glioma. Freshly resected tissues at surgery were immediately frozen in liquid nitrogen for subsequent total RNA extraction. Normal adult brains were obtained after informed consent from the patients with severe traumatic brain injury (TBI) who needed post-trauma surgery. RNA was extracted from tissues and glioma cell lines and astrocytes using TRIzol reagent (Invitrogen, Carlsbad, USA). Real-time quantification of miRNAs were by stem-loop RT-PCR. For the TaqMan-based real-time reverse transcription-polymerase chain reaction (RT-PCR) assays, the ABI 7300 HT Sequence Detection system (Applied Biosystem, Foster City, CA) was used. All the primers of miR-27b, and U6 for TaqMan miRNA assays were purchased from GenePharma Co., Ltd. (Shanghai, China). Real-time PCR was performed as previously described (12). The relative gene expression was calculated via the $2^{-\Delta\Delta Ct}$ method (13).

*Oligonucleotide transfection*. 2'-O-methyl (2'-O-Me) miR-27b was chemically synthesized by Shanghai GenePharma Co., Ltd. Sequences were as follows: 2'-O-Me-hsa-miR-27b 5'-GGUAAUCCCUGGCAAUGUGAU-3', and miRNA scrambled 5'-UUGUACUACACAAAAGUACUG-3'. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) at 70-90% confluence. Transfection complexes were prepared according to the manufacturer's instructions and added directly to the glioma cells to a final oligonucleotide concentration of 10 nmol/l. Transfection medium was replaced 8 h post-transfection.

*Cell growth assays*. The MTT assay was used to determine relative cell growth as follows. U87 and LN229 cells were plated at $10^4$ cells per well in 96-well plates with six replicate wells for each condition, transfected with oligonucleotides, and assayed 48 h post-transfection. Cell Growth Assay was performed by MTT (Sigma, St. Louis, MO) (14). The cell viability was determined at 540 nm absorbance using an enzyme-linked immunosorbent assay plate reader. All data points represent the mean of a minimum of six wells.

*Cell cycle analysis*. For cell cycle analysis by flow cytometry (FCM), transfected and control cells in the log phase of growth were harvested by trypsinization, washed with PBS, fixed with 75% ethanol overnight at 4˚C and then incubated with RNase at 37˚C for 30 min. Nuclei of cells were then stained with propidium iodide for 30 min. A total of $10^4$ nuclei were examined in a FACS Calibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and DNA histograms were analyzed by Modifit software (Becton Dickinson). Experiments were performed in triplicate.

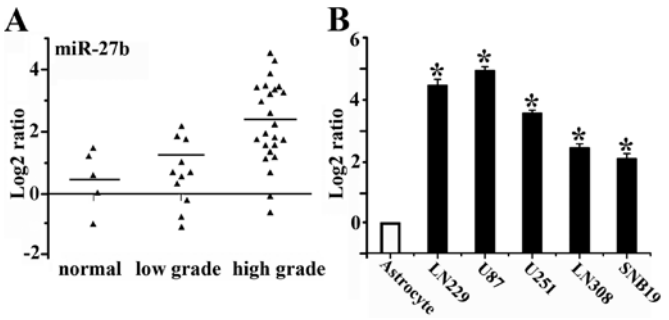


Figure 1. miR-27b was high expression in glioma samples and glioma cells. (A) MiR-27b expression in different grade glioma and normal brain tissues by real-time PCR. (B) qRT-PCR analysis show that LN229, U87, U251, LN308 and SNB19 glioma cells express higher levels of miR-27b compared with astrocyte. Data are mean ± SD) of three replicates. *P<0.05.

*Apoptosis assays*. U87 and LN229 cells were plated in 6-well plates and transfected with Oligonucleotide. The apoptosis ratio was analyzed 48 h post-transfection via using Annexin V FITC Apoptosis Detection kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Annexin VFITC and propidium iodide double stain was used to evaluate the percentages of apoptosis. Annexin $V^-$ and $PI^-$ cells were used as controls. Annexin $V^+$ and $PI^-$ cells were designated as apoptotic and Annexin $V^+$ and $PI^+$ cells were necrotic. Tests were repeated in triplicate.

*In vitro invasion assays*. Transwell membranes coated with Matrigel (BD Biosciences, San Jose, CA) were used to assay invasion of glioma cells *in vitro*. Transfected cells were plated at $5x10^4$ per well in the upper chamber in serum-free medium. FBS (20%) was added to the medium in the lower chamber. After incubating 24 h, noninvading cells were removed from the top well with a cotton swab while the bottom cells were fixed with 3% paraformaldehyde, stained with 0.1% crystal violet, and photographed in three independent x10 fields for each well. Three independent experiments were done and used to calculate fold migration relative to blank control while error was calculated as the SE.

*Luciferase reporter assay*. To evaluate the β-catenin/Tcf-4 transcriptional activity, we used a pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate). TOP-FLASH (with three repeats of the Tcf-binding site) or FOP-FLASH (with three repeats of a mutated Tcf-binding site) plasmids were transfected into cells treated with aspirin, as instructed by the suppliers. Luciferase activity was measured with the Dual-luciferase reporter assay system, with the Renilla luciferase activity as an internal control, 48 h after transfection.

*Western blot analysis*. U87 and LN229 cells were lysed in 1% Nonidet P-40 lysis buffer 48 h following control, scramble or miR-27b inhibitors transfection. SDS-PAGE was performed on 40 $\mu$g of protein from each sample, gels were transferred to PVDF membranes (Millipore, USA) and incubated with

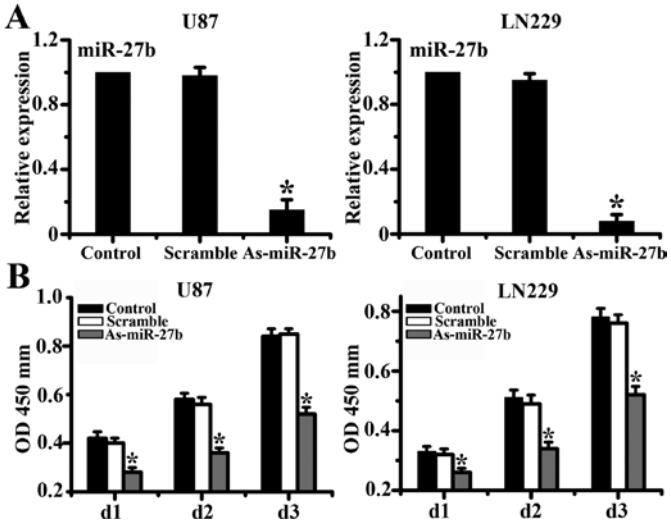


Figure 2. As-miR-27b suppresses U87 and LN229 proliferation. (A) miR-27b expression levels (normalized to U6 RNA) were significantly depressed by 86% (P<0.05) in U87/As-miR-27b and 92% (P<0.05) in LN229/As-miR-27b cells, relative to the scramble. (B) MTT assay showed that U87/As-miR-27b and LN229/As-miR-27b cells grew slower than cells transfected with the scramble and control. Data are the mean ± SD, n=3. *P<0.05.

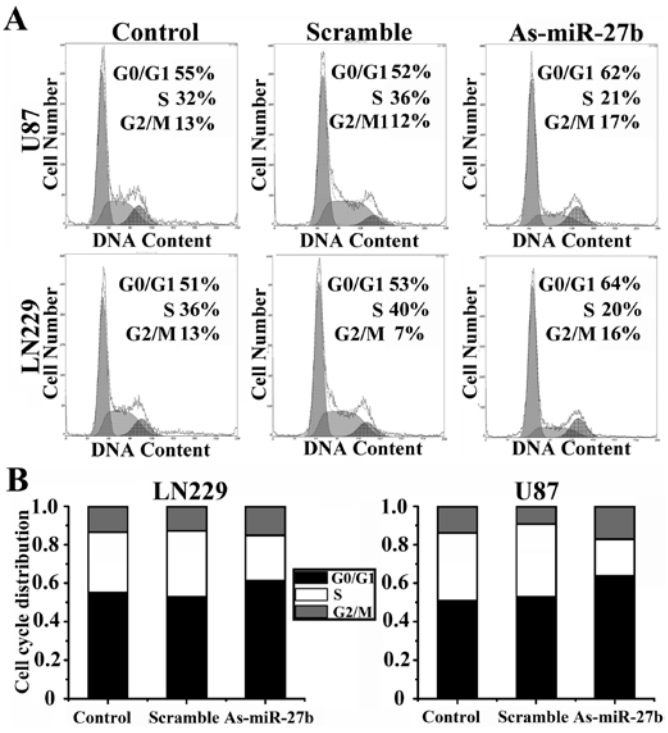


Figure 3. Low expression of miR-27b induced G0/G1 cell cycle arrest. Representative image of *in vitro* cell cycle assay of U87 and LN2291 after transfection with As-miR-27b and scramble. Percentage of cells in G0/G1, S and G2/M phases are indicated. Flow cytometry data represented as histograms reveal a significant increase in the percentage of cells in G0/G1 phase in the cell lines.

primary antibodies against c-myc, STAT3 (1:1000 dilution, CST, USA), Cyclin D1 (1:1000 dilution, Santa Cruz, USA), followed by incubation with an HRP conjugated secondary antibody (1:1000 dilution, Zymed, San Diego, CA). The specific protein was detected using a Super-Signal Protein Detection kit (Pierce, USA). After washing with stripping buffer, the membrane was reprobed with antibody against GAPDH (1:1000 dilution, Santa Cruz).

*Statistical analysis*. All tests were done using SPSS Graduate Pack 11.0 statistical software (SPSS, Chicago, IL). Descriptive statistics including the mean ± SE along with one-way ANOVAs were used to determine significant differences. P<0.05 was considered significant.

## Results

*MiR-27b is up-regulated in human glioma samples and glioma cell lines*. To investigate whether miR-27b was up-regulated in human gliomas and glioma cell lines as previously reported (11). We performed the TaqMan-based real-time stem-loop RT-PCR analyses. Our data showed similar results that miR-27b was strongly up-regulated in all grade glioma samples (WHO-II, WHO-III and WHO-IV glioma tissues) verses normal brain tissues, and miR-27b expression was positively correlated with tumor grade (Fig. 1A). We also examined expression levels of miR-27b in glioma cell lines (U87, LN229, SNB19 and LN308), and astrocytes as control (Fig. 1B). They demonstrated the same expression patterns as miR-27b in primary tumors and the normal tissues.

*Down-regulation of miR-27b inhibits glioma cell proliferation in vitro*. To explore the role of miR-27b in proliferation, we used a loss-of-function antisense approach. As-miR-27b oligonucleotide was used to knock down miR-27b expression in U87 and LN229 cells. RT-real-time PCR results determined that the relative expression levels of miR-27b in As-miR-27b treated glioma cells were successfully reduced compared with their control cells, respectively (Fig. 2A). Furthermore, MTT assay revealed that the knockdown of miR-27b significantly inhibited cell proliferation compared to the cells treated with control and scramble oligonucleotides (Fig. 2B). The cell cycle distribution of control and transfected cells was then analyzed by flow cytometry (Fig. 3). In U87 cells, the G0/G1 phase fraction of control and scramble treated cells was 55 and 52%, respectively. Administration of miR-27b inhibitor increased the percentage of cells in G0/G1 phase to 62%. In LN229 cells, the G0/G1 phase fraction of control and scramble treated cells was 51 and 53%, while miR-27b low-expression increased the G0/G1 phase to 64%. These results suggest that miR-27b expression can induce cell arrest in the G0/G1 phase, delay the progression of cell cycle, and inhibit cell proliferation.

*MiR-27b low-expression induces apoptosis in glioma cell lines*. To measure the effect of miR-27b expression on tumor cell apoptosis, we transfected U87 and LN229 cells with miR-27b inhibitors oligonucleotide or a scrambled control. After 48 h of transfection, apoptosis was measured by flow cytometry. Statistically significant increases in annexin $V^+$ apoptotic cells were observed in miR-27b inhibitor-treated group compared to untreated or scramble controls (Fig. 4).

*The cell invasive ability is depressed by miR-27b inhibitor*. To evaluate the impact of miR-27b expression on cell inva-

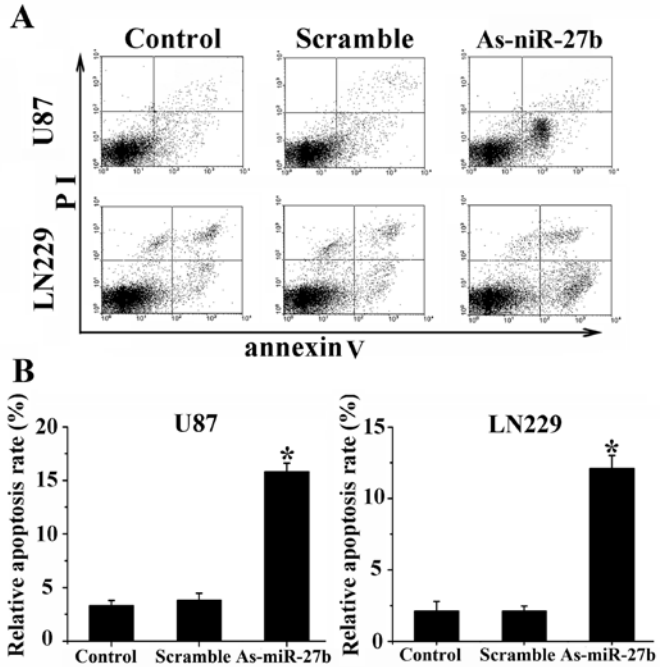


Figure 4. Low expression of miR-27b induces apoptosis. U87 and LN229 cells were treated with miR-27b inhibitor as described in Materials and methods. After 48 h of treatment, cells were harvested, double stained for Annexin V and propidium iodide and analyzed by flow cytometry. Early apoptotic cells are Annexin $V^+/PI^-$, late apoptotic cells are Annexin $V^+/PI^+$, necrotic cells are Annexin $V^-/PI^+$ and healthy cells are Annexin $V^-/PI^-$. A representative experiment of three performed is shown.

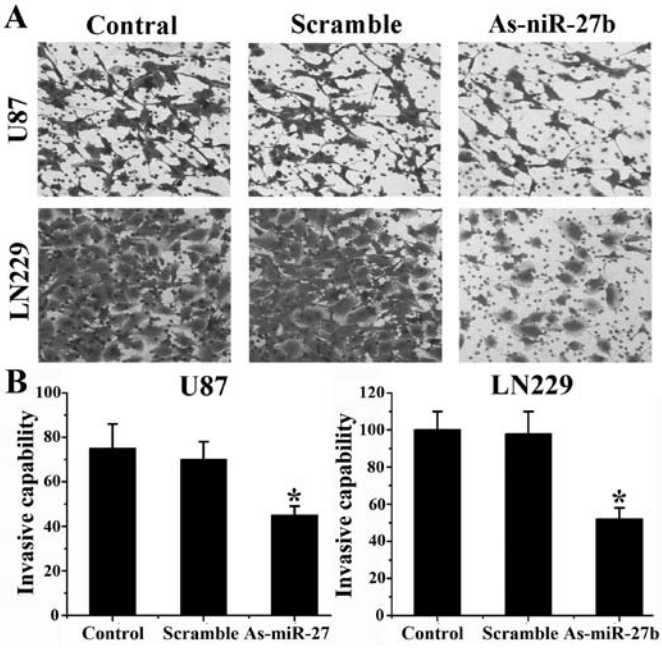


Figure 5. MiR-27b inhibitor impairs cellular invasive ability. U87 and LN229 cell invasion was determined by the Transwell invasion assay. Quantification of the number of cells invades through the matrigel into the lower surface of the polycarbonic membrane. Cell invasion was decreased miR-27b inhibitor-treated cells compared with control and scramble groups ($^*p<0.05$).

sion, U87 and LN229 cells were treated with oligonucleotide and placed in a transwell chamber. Cell invasive potential was determined on the basis of the ability of cells to invade a matrix

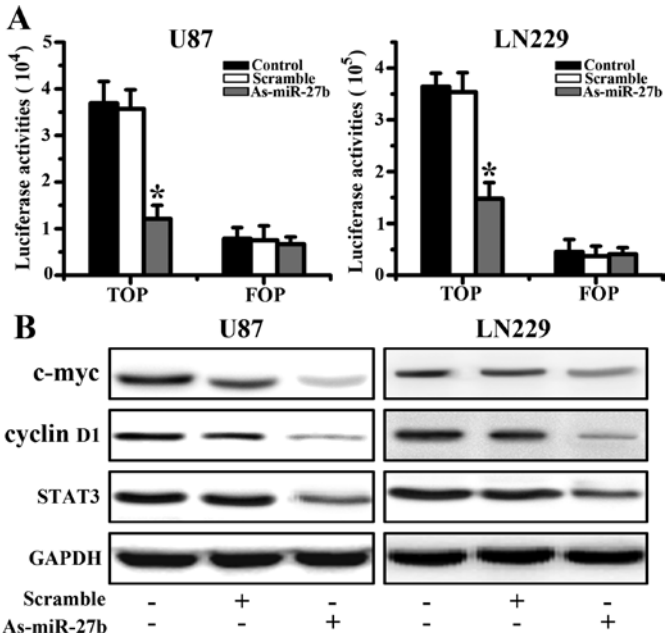


Figure 6. Impact of miR-27b inhibitor on β-catenin/Tcf-4 transcription activities. U87 and LN229 cells were treated with As-miR-27b, as described in Materials and methods. TOP/FOP flash luciferase activities were detected 48 h following treatment. Furthermore, c-myc, cyclinD1, STAT3 and GAPDH expression were determined by Western blotting. Data are from one of three representative experiments.

barrier consisting of laminin and type IV collagen, which are the major components of the basal membrane. The number of U87 and LN229 cells invading through the matrigel following miR-27b inhibitor treatment was significantly decreased from those of the control and the scramble treated cells (Fig. 5). Our experiments demonstrate that miR-2b low-expression inhibits the invasive ability of U87 and LN229 glioma cells *in vitro*.

*MiR-27 low-expression reduces transcription activities of β-catenin/Tcf-4*. More and more studies have reported that β-catenin/Tcf-4 pathway played an important role in the pathogenesis of glioma (15,16). Herein, we wanted to detect the transcription activities of β-catenin/Tcf-4 in glioma after treatment by miR-27b inhibitor. As expected, TOP flash luciferase activities were significantly suppressed, while FOP flash luciferase activities were not obviously changed. Further, miR-27b inhibitor oligonucleotides reduced CyclinD1, STAT3 and c-myc expression which were downstream of β-catenin/Tcf-4 pathway (Fig. 6). These data suggested that the carcinogenesis activity of miR-27b in glioma cells may be regulated by the β-catenin/Tcf-4 pathway.

## Discussion

In our previous studies, we profiled miRNA expression in five glioma cell lines, one astrocytoma cell line, and normal brain tissue. Our data revealed that of the 453 miRNAs analyzed, >2-fold overexpression was observed for 8 miRNAs (1.84%), and >2-fold reduction was observed in 18 (3.68%). MiR-27b was one of up-regulated microRNAs. Herein, we confirmed that miR-27b was highly expressed both in glioma samples and glioma cell lines. Furthermore, Down-regulation of miR-27b

results in glioma cell growth inhibition, invasion reduction and apoptosis induction. Altogether, these findings document that the expression of miR-27b was up-regulated significantly in glioma and may play a critical role in the pathogenesis of glioma.

MicroRNAs have been shown to be important regulators of cellular proliferation, differentiation and the cell cycle in glioma (17,18). Chan *et al* reported that miR-21 levels were markedly elevated, and down-regulated miR-21 expression in cultured glioblastoma cells could lead to induction of apoptotic cell death (8). Further, they used bioinformatics analysis and biochemistry to confirm that miR-21 binds and silences the tumor suppressor gene PDCD4. Zhang *et al* used a similar approach indicating that miR-221/222 inhibit cell apoptosis by targeting pro-apoptotic gene PUMA in human glioma cells (19). Recently, the research team of Krichevsky validated that the important role of miR-10b in gliomagenesis, and reveal a novel mechanism of miR-10b mediated regulation (20). Following the approach of Chan *et al*, we used 2'-O-methyloligonucleotides to knockdown the expression of miR-27b in human glioblastoma cells (8). After transfection, our results revealed that the proliferation of glioma cells was inhibited by MTT and cell cycle analysis. Meanwhile, the annexin V/PI and transwell assay demonstrated that down-regulation of miR-27b can significantly suppress invasion and induce apoptosis. Thus, knockdown of miR-27b in glioma is a potential therapy in future.

MiR-27b belongs to the miR-23b~27b~24-1 cluster which is localized on chromosome 19p13. Rogler *et al* identified that miR-23b cluster miRNAs were up-regulated during late fetal development in mouse by miRNA profiling. In addition, bioinformatic and luciferase reporter assays confirmed that Smads were the direct target of the miR-23b cluster (21). Crist *et al* showed that miR-27b was expressed in the differentiating skeletal muscle of the embryonic myotome and in activated satellite cells of adult muscle. Both *in vitro* and *in vivo* experiments validated that miR-27b regulated Pax3 protein levels and this down-regulation ensures rapid and robust entry into the myogenic differentiation program (22). So far, there are no function reports of miR-27b in cancer. We are the first to delineate the expression and effect of miR-27b in glioma. TOP/FOP luciferase reporter assays and Western blot confirmed that the function of miR-27b was possibly mediated by β-catenin/Tcf-4 pathway. Our unpublished studies demonstrated miR-27b can increase the sensitivity of tomozolomide in glioma.

In conclusion, we established that miR-27b was up-regulated in glioma samples and glioma cell lines. Down-regulation of miR-27b inhibited the proliferation, induced apoptosis and suppressed the invasion of glioma. Furthermore, the effect of miR-27b in glioma might be mediated by β-catenin/Tcf-4 activities. Thus, knockdown of miR-27b is a potential approach in future.

## Acknowledgements

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