

# MicroRNA-216b inhibits cell proliferation and invasion in glioma by directly targeting metadherin

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**Abstract.** Glioma is a well-known aggressive and malignant brain tumor, and accounts for ~30% of all brain and central nervous system tumors. A number of studies have indicated that the abnormal expression of specific microRNAs (miR) serves vital roles in the tumorigenesis and tumor development of human cancer, including glioma. miR-216b has been studied in a number of types of cancer. However, the expression pattern, molecular function and underlying mechanisms of miR-216b in glioma remain unclear. In the present study, it was demonstrated that the level of miR-216b was significantly decreased in glioma tissues and cell lines compared with matched normal tissues and primary normal human astrocytes. The reduced miR-216b expression level was correlated with the Karnofsky Performance Score and the World Health Organization grade of gliomas. Upregulation of miR-216b repressed cell proliferation and invasion in glioma. Additionally, metadherin (MTDH) was identified as a direct target gene of miR-216b in glioma. MTDH expression was demonstrated to be significantly upregulated and inversely associated with miR-216b expression in glioma specimens. MTDH knockdowns could simulate the cellular conditions induced by miR-216b overexpression in glioma cells. In addition, miR-216b regulated phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN/protein kinase B signaling pathways in glioma. These results suggested that miR-216b acted as a tumor suppressor in glioma by directly targeting MTDH and that the miR-216b/MTDH axis may be an effective therapeutic target for the treatment of patients with this disease.

## Introduction

Glioma, a well known aggressive and malignant brain tumor, accounts for ~30% of all brain and central nervous system tumors and 80% of malignant tumors in the brain (1). Glioma can be classified as low-grade or high-grade tumors, depending on the 2007 World Health Organization (WHO) grading system (2). A number of risk factors contributing to the development of glioma have been identified, including radiation, use of exogenous hormones, consumption of coffee and tea, smoking status and physical conditions (3,4). Despite improvements in the therapeutic treatment strategies for glioma, including surgery, radiotherapy, chemotherapy, gene therapy, immunotherapy and other novel biological therapies, patients with this disease exhibit a median survival of 15 months (5). The primary reasons for the poor prognosis of gliomas is recurrence and local invasion of the tumor into normal brain tissues (6). Therefore, it is urgent to fully understand the molecular and cellular mechanisms of glioma and develop more effective strategies for this malignancy.

microRNAs (miRs) are a group of endogenous, non-coding, short (~22 nucleotides) RNAs that negatively regulate gene expression by base pairing with the 3'-untranslated regions (3'UTRs) of their target genes, resulting in either mRNA degradation or suppression of translation (7). Theoretically, a single miR can modulate a number of target genes simultaneously, while a single gene could be regulated by multiple miRs (8). Numerous studies have demonstrated that miRs are involved in complex regulatory networks that are implicated in a multitude of cellular processes, including cell proliferation, cell cycle progression, apoptosis, differentiation, invasion, migration and metastasis (9-11). In addition, the abnormal expression of specific miRs serves vital roles in tumorigenesis and tumor development in human cancer, as miRs target a number of tumor suppressors and oncogenes (12). Expression levels of certain miRs could serve as diagnostic and prognostic indicators for patients with cancer (13). For example, miR-140 is downregulated in glioma and low expression is associated with a low WHO grade and Karnofsky Performance Score (KPS) in patients with glioma. Upregulation of miR-140 significantly inhibited glioma cell proliferation, migration and invasion through directly targeting disintegrin and metalloproteinase domain-containing protein 9 (14). Therefore, miRs have potential as novel therapeutic targets for the diagnosis and treatment of human cancer.

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miR-216b has been demonstrated to serve key roles in the tumorigenesis and tumor development in several types of human cancer (15-17). However, the detailed regulatory mechanisms of miR-216b in glioma remain unknown. Therefore, the present study aimed to investigate the expression level and biological roles of miR-216b in glioma, as well as its underlying molecular mechanisms.

## Materials and methods

**Tissue samples.** A total of 48 paired human glioma tissues and matched normal tissues were collected from patients who underwent surgical resection at the Department of Neurosurgery, The Second Affiliated Hospital of Nanchang University (Nanchang, China) between June 2014 and January 2016 and their details are presented in Table I. None of these patients were treated with radiotherapy, chemotherapy, gene therapy, immunotherapy or other novel biological therapies. All tissues were flash-frozen in liquid nitrogen immediately following collection and then stored at -80°C until RNA extraction. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. Written informed consent was also acquired from each patient.

**Cell lines.** A total of five glioma cell lines (U87, U251, U373, LN18, A172) and primary normal human astrocytes (NHA) were purchased from the American Type Culture Collection (Manassas, VA, USA). The origin of the U87 cell line is unknown, but it is a likely glioblastoma cell line (18). U373 cell line, known as a U251 derivative (19), was acquired from the National Infrastructure of Cell line Resource (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

**Cell transfection.** A mature miR-216b mimic and an miRNA mimic negative control (miR-NC) were obtained from Chang Jing Bio-Tech, Ltd. (Changsha, China). The miR-216b mimic sequence was 5'-AAAUCUCUGCAGGCAA AUGUGA-3' and the miR-NC sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. Small interfering RNA targeting metadherin (MTDH siRNA) and its NC siRNA were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The MTDH siRNA sequence was 5'-GCTGTTCGAACACCTCAA-3' and the NC siRNA sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. For transfection, cells were seeded into 6-well plates at a density of 60-70% confluence. Cells were transfected with an miR-216b mimic (100 pmol), miR-NC (100 pmol), MTDH siRNA (100 pmol) or NC siRNA (100 pmol) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. At 48 h post-transfection, cells were collected and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to evaluate the transfection efficiency.

**RT-qPCR.** Tissue samples and cells were subjected to RNA isolation using the TRIzol® reagent (Invitrogen; Thermo

Table I. Association between miR-216b and the clinicopathological factors of patients with glioma.

Clinicopathological factors	No. of cases	miR-216b expression		P-value
		Low (n)	High (n)	
Sex				0.658
Male	28	15	13	
Female	20	12	8	
Age, years				0.715
<55	22	13	9	
≥55	26	14	12	
KPS				0.018
≥80	25	10	15	
<80	23	17	6	
WHO grade				0.022
I-II	23	9	14	
III	25	18	7	

WHO, World Health Organization; KPS, Karnofsky performance score; miR, microRNA.

Fisher Scientific, Inc.) following the manufacturer's protocol. The concentration and purity of total RNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). To measure miR-216b expression, complementary DNA (cDNA) was synthesized using a TaqMan® MicroRNA Reverse Transcription kit and qPCR was conducted with a TaqMan® MicroRNA Assay kit (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec. The primers were designed as follows: miR-216b, 5'-AAATCTCTGCAGGCAAATGTGA-3' (forward) and 5'-GTGCAGGTCCGAGGT-3' (reverse); U6, 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCA-3' (reverse). To detect MTDH mRNA expression, cDNA was synthesized using Moloney-murine leukemia virus reverse transcription system (Promega Corporation, Madison, WI, USA), followed by qPCR using SYBR Premix Ex Taq (Takara, Dalian, China). The cycling conditions were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. The primers were as follows: MTDH, 5'-TGTTGAAGTGGCTGAGGG-3' (forward) and 5'-CAGGAAATGATGCGGTTG-3' (reverse); and GAPDH, 5'-GGTGAAGGTCGGAGTCAACG-3' (forward) and 5'-CAAGTTTGCATGGATGHACC-3' (reverse). The miR-216b and MTDH mRNA expression was normalized to those of U6 and GAPDH, respectively, using the 2<sup>-ΔΔC<sub>q</sub></sup> method (20).

**MTT assay.** Cell proliferation was evaluated using an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Transfected cells were harvested at 24 h post-transfection and then plated into 96-well plates at a density of 2,000 cells/well.

Cells were incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> for 1, 2, 3 and 4 days. At specific time points, the MTT assay was performed according to the manufacturer's protocol. A total of 10 µl MTT reagent (5 mg/ml) was added into each well and the plates were incubated at 37°C for an additional 4 h. The culture medium containing the MTT solution was removed and formazan crystals were dissolved in 150 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). Cellular proliferation was determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by measuring the absorbance of the converted dye at 490 nm. All experiments were performed in triplicate.

**Transwell invasion assay.** Transwell filters (diameter 12 mm; pore size 8-µm; EMD Millipore, Billerica, MA, USA) coated with Matrigel® (BD Biosciences, San Jose, CA, USA) were utilized to assess the invasive ability of cells. A total of 48 h following transfection, cells were collected and resuspended in FBS-free culture medium. A total of 1x10<sup>5</sup> cells/400 µl were plated into the upper chamber, while 600 µl culture medium containing 20% FBS was added into the lower chamber. The plates were incubated at 37°C for 48 h. Non-invasive cells were removed using a cotton swab. Invasive cells were fixed with 100% methanol at room temperature for 10 min, stained with 1% crystal violet at room temperature for 10 min, washed at room temperature for three times and dried in air. Images of the stained cells were captured and counted under a microscope (magnification, x200; IX53; Olympus Corporation, Tokyo, Japan) in three independent fields for each Transwell filter.

**The predication of miR-216b targeting gene.** The target genes of miR-216b were predicted using PicTar (pictar.mdc-berlin.de/) and TargetScan (www.targetscan.org).

**Luciferase reporter assay.** For the luciferase reporter assay, the pMIR-MTDH-3'UTR wild-type (Wt) and pMIR-MTDH-3'UTR mutant-type (Mut) reporter vectors were synthesized by Chang Jing Bio-Tech, Ltd. 293T cells were seeded into 24-well plates at a density of 50-60% confluence and co-transfected with an miR-216b mimic or miR-NC, and the pMIR-MTDH-3'UTR Wt or the pMIR-MTDH-3'UTR Mut, using Lipofectamine 2000. Following incubation for 48 h at 37°C in 5% CO<sub>2</sub> for 48 h, the luciferase activity was detected using the Dual-Luciferase® Reporter Assay system (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

**Western blotting.** Transfected cells were harvested at 72 h post-transfection and total protein was extracted using a radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), which contained a protease inhibitor. The concentration of total protein was measured using the bicinchoninic assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 µg) were loaded onto 10% SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes (EMD Millipore), blocked with 5% skimmed milk with TBS containing 0.1% Tween-20 (TBST) at room temperature for 1 h and then incubated with the primary antibodies at 4°C overnight. The primary antibodies used in the

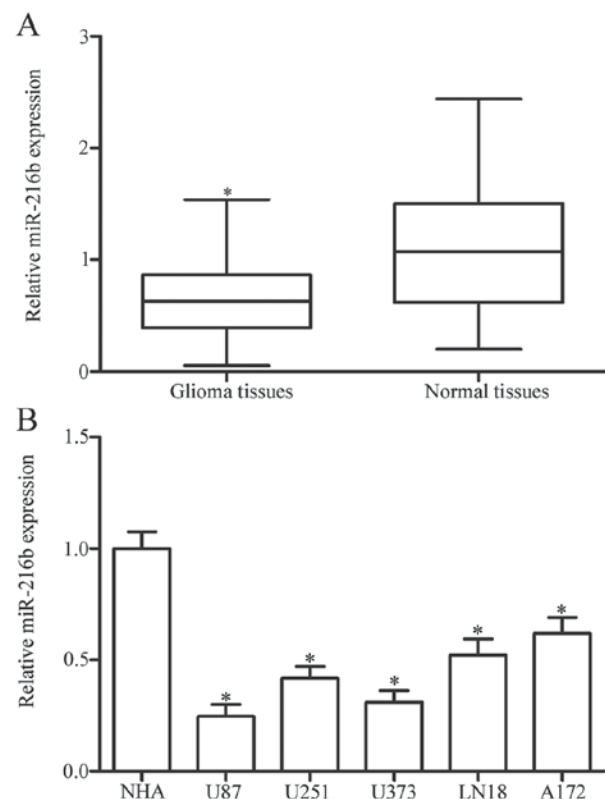


Figure 1. miR-216b was lowly expressed in glioma tissues and cell lines. (A) Relative expression of miR-216b was determined in glioma tissues and matched normal tissues. (B) miR-216b expression in five glioma cell lines (U87, U251, U373, LN18 and A172) and primary NHA. \*P<0.05. NHA, normal human astrocytes; miR-216b, microRNA-216b.

present study included mouse anti-human MTDH monoclonal antibody (cat no. sc-517220; 1:1,000) and mouse anti-human GAPDH monoclonal antibody (cat no. sc-32233 1:1,000), rabbit anti-human polyclonal protein kinase B (AKT; cat no. sc-8312; 1:1,000), mouse anti-human monoclonal phosphorylated (p)-AKT (cat no. sc-514032; 1:1,000) and mouse anti-human monoclonal phosphatase and tensin homolog (PTEN; cat no. sc-7974; 1:1,000) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were washed with TBST three times and probed with the corresponding horseradish peroxidase-conjugated secondary antibody (sc-2005; 1:5,000 dilution; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Finally, the protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) and analyzed using AlphaEase™ FC software (version 4.0.1; ProteinSimple; Bio-Techne, Minneapolis, MN, USA). GAPDH was used as an internal control.

**Statistical analysis.** Statistical analyses were performed using SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA). All results were expressed as the mean ± standard deviation or box plots. The Student's t-test and one-way analysis of variance (ANOVA) plus multiple comparisons were used to analyze significant differences between groups. The post hoc test used after ANOVA was Student-Newman-Keuls. The correlation between miR-216b expression and the clinicopathological factors was analyzed by the  $\chi^2$  test. Spearman's rank correlation

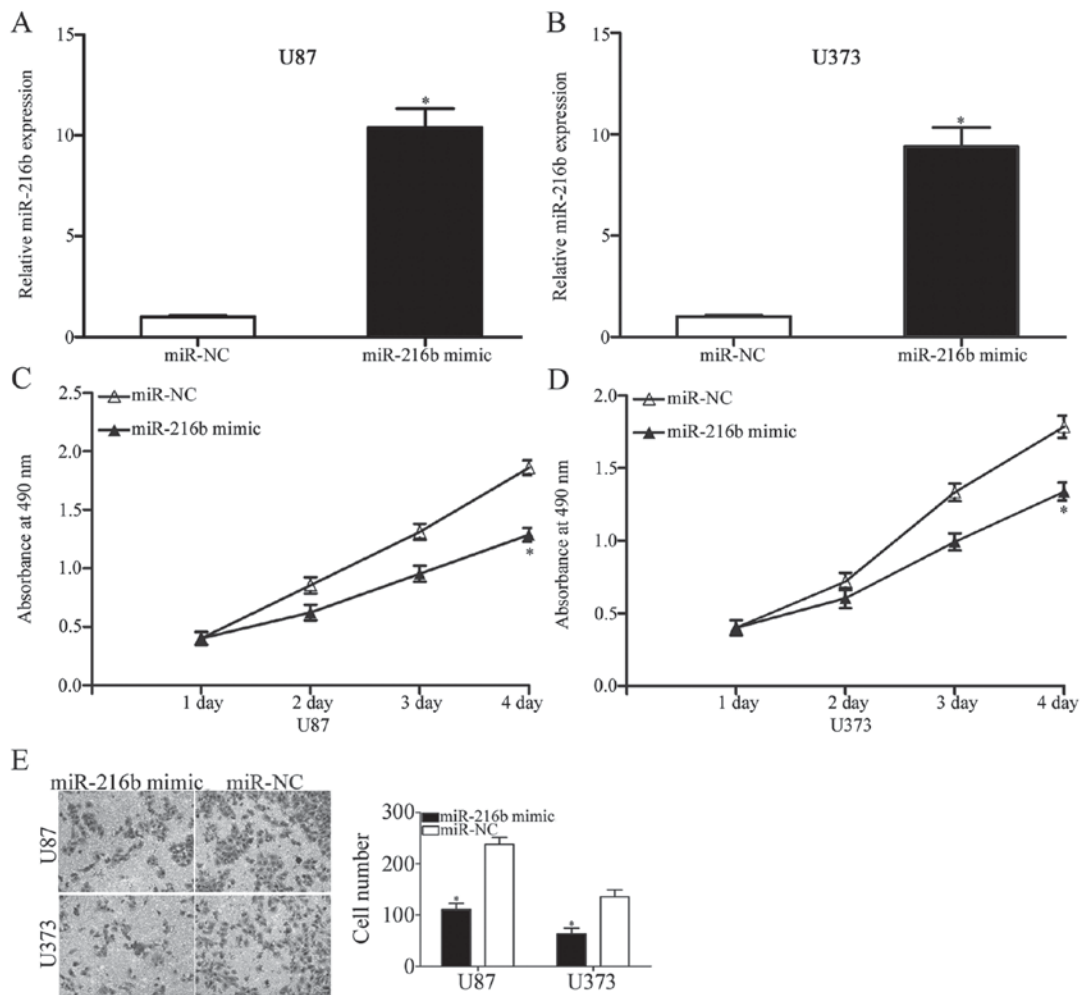


Figure 2. miR-216b inhibits U87 and U373 cell proliferation and invasion. Reverse transcription-quantitative polymerase chain reaction was performed to measure miR-216b expression in (A) U87 and (B) U373 cells following transfection with an miR-216b mimic or miR-NC. Cell proliferation in (C) U87 and (D) U373 cells following transfection with an miR-216b mimic or miR-NC and (E) invasion were assessed. Absorbance at 490 nm represents the optical density. \* $P<0.05$ . miR-NC, microRNA mimic negative control.

coefficient analysis was adopted to evaluate the association between miR-216b and MTDH mRNA expression.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

*miR-216b is downregulated in glioma tissues and cell lines.* miR-216b expression was measured in glioma tissues and matched normal tissues using RT-qPCR. The results demonstrated that the expression level of miR-216b was significantly decreased in the glioma tissues compared with the matched normal tissues ( $P<0.05$ ; Fig. 1A). miR-216b expression in five glioma cell lines (U87, U251, U373, LN18 and A172) and primary NHA was examined. As presented in Fig. 1B, all glioma cell lines exhibited reduced expression of miR-216b in comparison with NHA ( $P<0.05$ ). These results supported the hypothesis that reduced miR-216b may be involved in glioma formation and progression.

*Low expression of miR-216b is associated with the clinicopathological features of glioma.* The association between miR-216b expression and the clinicopathological features of

patients with glioma was analyzed. All glioma tissue samples were divided into two subgroups according mean value (0.64); a low miR-216b group (27 cases) and a high miR-216b group (21 cases). As demonstrated in Table I, low expression of miR-216b was associated with a low KPS ( $P=0.018$ ) and WHO grade ( $P=0.022$ ) in patients with glioma. However, no association was observed between miR-216b expression and the sex ( $P=0.658$ ) or age ( $P=0.715$ ) of the patients.

*miR-216b inhibits glioma cell proliferation and invasion.* To investigate the effects of miR-216b on glioma initiation and progression, an miR-216b mimic was introduced into U87 and U373 cells. At 48 h post-transfection, RT-qPCR was performed to evaluate the efficiency of overexpression and it was demonstrated that miR-216b was upregulated in U87 and U373 cells following transfection with an miR-216b mimic compared with cells transfected with miR-NC ( $P<0.05$ ; Fig. 2A and B). An MTT assay was conducted to investigate the effect of miR-216b on glioma cell proliferation. As demonstrated in Fig. 2C and D, ectopic expression of miR-216b suppressed U87 and U373 cells proliferation ( $P<0.05$ ). A Transwell invasion assay was used to evaluate the roles of miR-216b on



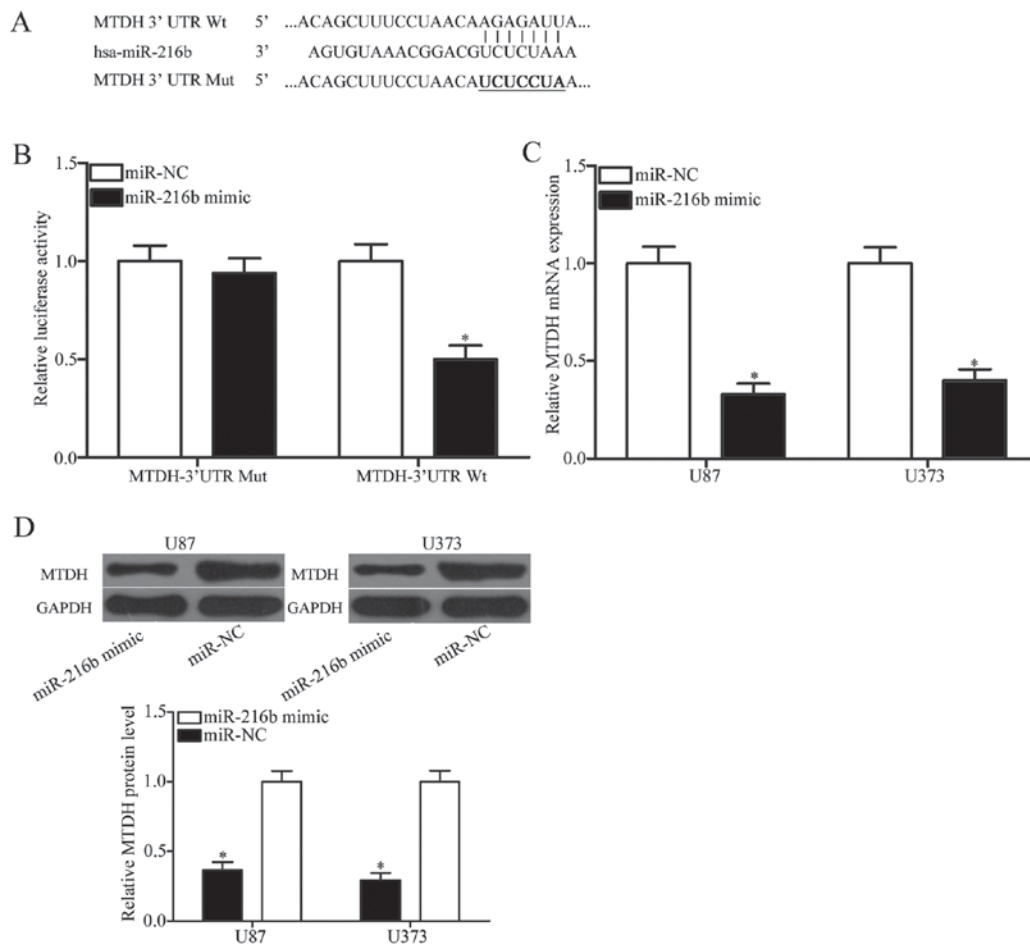


Figure 3. MTDH is a direct target of miR-216b in glioma. (A) Predicted binding sites for miR-216b in the 3'UTR of MTDH and the mutations in the binding sites. (B) Luciferase reporter assay to detect the relative luciferase activities in 293T cells following co-transfection with pMIR-MTDH-3'UTR Wt or pMIR-MTDH-3'UTR Mut and an miR-216b mimic or miR-NC. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to measure MTDH (C) mRNA and (D) protein expression in U87 and U373 cells following transfection with an miR-216b mimic or miR-NC. \* $P < 0.05$  vs. NHA. MTDH, metadherin; miR-216b, microRNA-216b; miR-NC, miR mimic negative control; 3'UTR, 3'untranslated region; Wt, wild type; Mut, mutant; hsa, *Homo sapiens*.

glioma cell metastasis. The results indicated that expression of miR-216b decreased the invasive ability of U87 and U373 cells (Fig. 2E,  $P < 0.05$ ). These results suggested that miR-216b exerts a tumor suppressor role in glioma.

**MTDH is a direct target of miR-216b in glioma.** Based on bioinformatic analysis using PicTar and TargetScan, a potential list of target genes was predicated. Among these genes, MTDH was of interest (Fig. 3A). MTDH is upregulated in glioma (21) and involved in glioma occurrence, and development (22) indicating that MTDH may be a direct target of miR-216b in glioma. To confirm whether MTDH was a direct target of miR-216b, a luciferase reporter assay was performed. 293T cells were transfected with luciferase reporter vectors, together with an miR-216b mimic or miR-NC. The results demonstrated that the luciferase activity was reduced by the co-transfection with an miR-216b mimic and pMIR-MTDH-3'UTR Wt ( $P < 0.05$ ; Fig. 3B); however, co-transfection of an miR-216b mimic and pMIR-MTDH-3'UTR Mut did not affect the luciferase activity in 293T cells. To further determine the regulatory effects of miR-216b on endogenous MTDH expression, RT-qPCR

and western blotting was performed in U87 and U373 cells following transfection with an miR-216b mimic or miR-NC. As demonstrated in Fig. 3C and D, upregulation of miR-216b suppressed MTDH mRNA and protein expression in U87 and U373 cells (all  $P < 0.05$ ). These results demonstrated that MTDH is a direct target gene of miR-216b in glioma.

**miR-216b was negatively correlated with MTDH expression in glioma tissues.** As miR-216b was lowly expressed in glioma tissues and miR-216b inhibited glioma cell proliferation and invasion by negative regulation of MTDH, it was hypothesized that the expression level of miR-216b may be inversely correlated with miR-216b expression in glioma tissues. MTDH expression was examined in glioma tissues and matched normal tissues using RT-qPCR. As demonstrated in Fig. 4A, elevated expression of MTDH was observed in glioma tissues compared with the matched normal tissues ( $P < 0.05$ ). Furthermore, Spearman's rank correlation coefficient analysis indicated an inverse correlation between miR-216b and MTDH mRNA expression in glioma tissues ( $P = 0.001$ ;  $r = -0.4597$ ; Fig. 4B). These results confirmed that MTDH is a direct target of miR-216b in glioma.

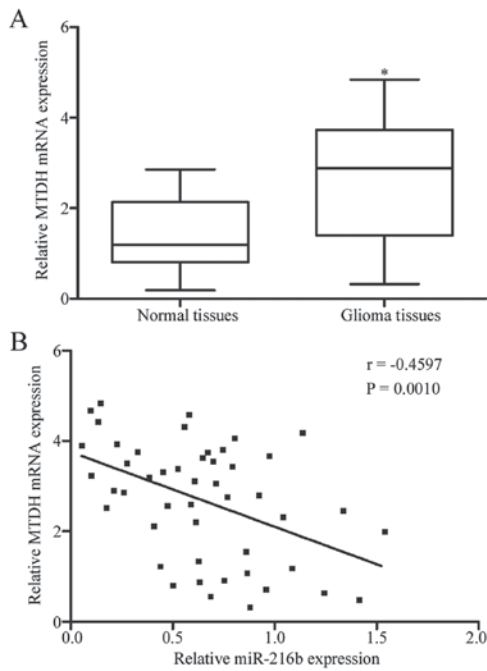


Figure 4. An inverse correlation between miR-216b and MTDH mRNA expression in glioma tissues. (A) The mRNA expression levels of MTDH in glioma tissues were determined by reverse transcription-quantitative polymerase chain reaction. (B) Spearman's rank correlation coefficient analysis of the relative expression levels of miR-216b and MTDH mRNA expression glioma specimens. \* $P < 0.05$ . MTDH, metadherin; miR-216b, microRNA-216b.

*miR-216b exerts its tumor suppressive roles by downregulating MTDH expression.* As MTDH was identified as a direct

target of miR-216b, the roles of MTDH in cell proliferation and invasion in glioma were investigated. MTDH expression was silenced using MTDH siRNA and confirmed by western blotting ( $P < 0.05$ ; Fig. 5A). MTT and Transwell invasion assays were used to assess the effects of MTDH-knockdown on cell proliferation and invasion in glioma. The results demonstrated that the inhibition of MTDH expression by transfection with MTDH siRNA decreased U87 and U373 cell proliferation ( $P < 0.05$ ; Fig. 5B and C) and invasion ( $P < 0.05$ ; Fig. 5D). These results suggest that miR-216b inhibited glioma cell proliferation and invasion, partially through downregulation of MTDH expression.

*miR-216b inhibits the PTEN/AKT signaling pathway.* Previous studies demonstrated that MTDH could negatively regulate PTEN expression via blocking its transcription (23,24). Therefore, PTEN, AKT and p-AKT expression was measured in U87 and U373 cells following transfection with an miR-216b mimic or miR-NC. As demonstrated in Fig. 6, upregulation of miR-216b enhanced PTEN expression and reduced p-AKT expression, whereas miR-216b did not affect AKT expression in U87 and U373 cells ( $P < 0.05$ ). These results indicated that miR-216b is involved in PTEN/AKT signaling pathway through regulation of MTDH.

## Discussion

The heterogeneity, aggressive nature and angiogenic behavior of glioma results in high morbidity, high recurrence rate, high mortality and low cure rate, and therefore results in a poor prognosis and short survival time (6,25,26). A number of

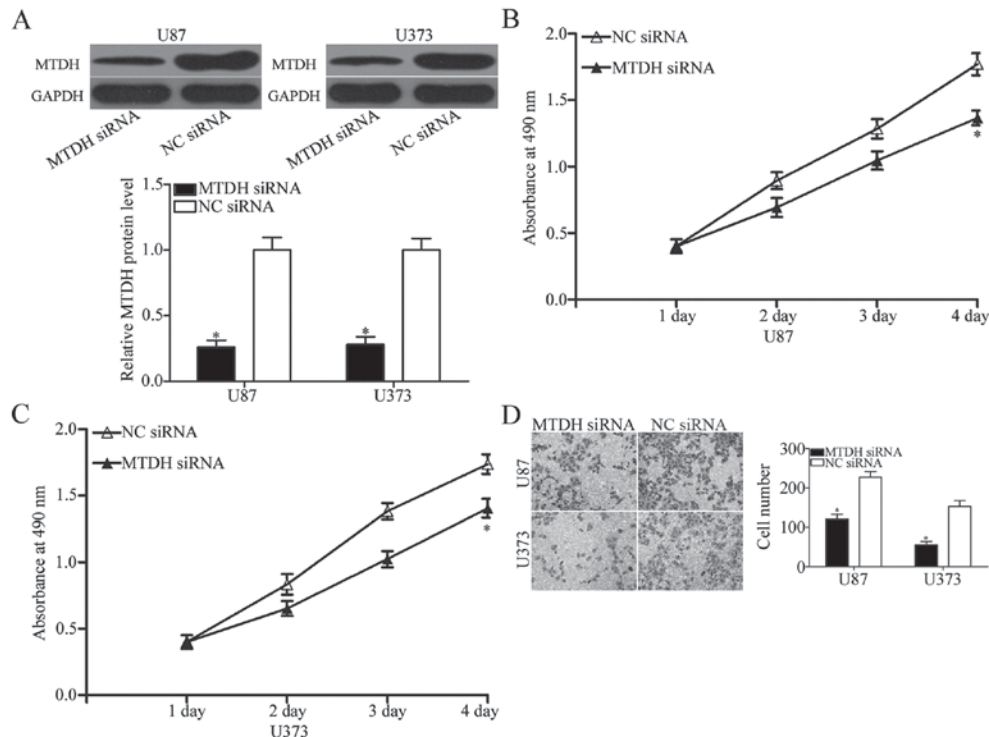


Figure 5. MTDH knockdown repressed U87 and U373 cells proliferation and invasion. (A) Expression levels of MTDH in U87 and U373 cells transfected with MTDH siRNA or NC siRNA were detected using western blotting. Cell proliferation in (B) U87 and (C) U373 cells and (D) invasion were assessed following transfection with MTDH siRNA or NC siRNA. \* $P < 0.05$ . MTDH, metadherin; siRNA, small interfering RNA; NC, negative control.

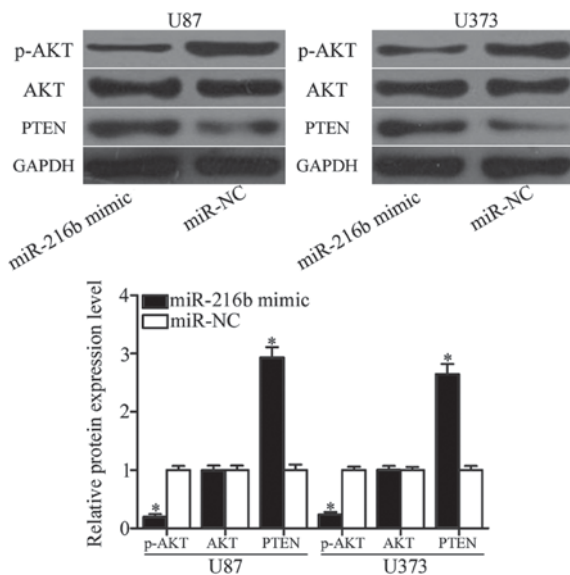


Figure 6. Quantified western blot analysis of the effect of restoration of the expression of miR-216b on PTEN AKT and p-AKT expression in U87 and U373 cells. miR-216b, microRNA-216b; PTEN, phosphatase and tensin homolog; p-AKT, phosphorylated-protein kinase B; miR, microRNA.

studies have demonstrated that miRs serve important roles in the formation and progression of glioma as oncogenes or tumor suppressor genes through negative regulation of their targets (27-29). miRs are considered to be potential targets for diagnosis, therapy and prognosis in glioma.

Previous studies have demonstrated that miR-216b is abnormally expressed in several types of human cancer. For instance, in nasopharyngeal carcinoma, miR-216b was down-regulated in tumor tissues and cell lines. Reduced miR-216b expression was strongly correlated with advanced clinical stage and lymph node metastasis (15). Liu *et al* (16) reported that miR-216b was lower in hepatocellular carcinoma tissues and was associated with tumor volume, hepatitis B virus (HBV) infection, HBV DNA level and vascular invasion in patients with this disease. Egeli *et al* (30) demonstrated that miR-216b expression was low in pancreatic ductal adenocarcinoma. Expression level of miR-216b was correlated with aggressive tumor characteristics and shortened disease-free survival of patients with pancreatic ductal adenocarcinoma. Aberrant downregulation of miR-216b was also observed in breast (31,32) and gastric cancer (33). However, the expression pattern of miR-216b in glioma remains unclear. RT-qPCR was used to examine miR-216b expression in glioma tissues and cell lines. Results demonstrated that expression level of miR-216b was reduced in glioma tissues and cell lines. Low miR-216b expression was correlated with the KPS and WHO grade of gliomas. These results suggested that miR-216b appeared to be involved in carcinogenesis and cancer progression.

miR-216b has been investigated in the development of a number of human cancers. In nasopharyngeal carcinoma, restoration of expression of miR-216b produced a suppressive effect on cell proliferation, invasion *in vitro*, as well as tumor growth *in vivo* (15,17). In breast cancer, the introduction of miR-216b inhibited cell proliferation, migration, invasion and induced apoptosis (31,32). In hepatocellular carcinoma,

miR-216b overexpression suppressed cell growth and metastasis and enhanced cell cycle arrest, and apoptosis (16,34). In gastric cancer, upregulation of miR-216b attenuated cell proliferation and cell cycle progression (33). In the present study, it was demonstrated that ectopic expression of miR-216b repressed glioma cell proliferation and invasion *in vitro*. These results suggested that miR-216b was characterized as an important tumor suppressor and may therefore serve as a potential therapeutic target for the treatment of these types of cancer.

miRs are known to regulate target mRNAs through directly binding to the 3'UTR of target gene, (9). Several oncogenes have been identified as direct targets of miR-216b, including protein kinase C  $\alpha$  (17), GTPase KRAS (15) in nasopharyngeal carcinoma, insulin-like growth factor 2 mRNA-binding protein 2 (16), forkhead box protein M1 (34) in hepatocellular carcinoma and histone deacetylase 8 (33) in gastric cancer, syntenin-1 (35) in breast cancer, and fibroblast growth factor receptor 1 (30) in pancreatic cancer. A previous study also indicated that expression level of miR-216b could be regulated by C/EBP-homologous protein 10/growth arrest and DNA damage-inducible protein 153 (36). In the present study it was confirmed that MTDH was a novel, direct and functional target of miR-216b in glioma. Bioinformatic analysis was performed to predicate the candidate targets of miR-216b. MTDH was selected for further investigation. A luciferase reporter assay revealed that miR-216b was able to directly target the 3'UTR of MTDH. In addition, MTDH mRNA and protein expression could be negatively regulated by miR-216b in glioma cells. Furthermore, the negative association between miR-216b and MTDH mRNA expression in glioma tissues further confirmed that MTDH is a direct target of miR-216b. MTDH knockdown mimicked the effects of miR-216b overexpression on glioma cell proliferation and invasion.

MTDH, also known as astrocyte elevated gene-1, was first identified in human fetal astrocytes in 2002 (37). MTDH is located at chromosome 8q22 and encodes a 582-amino acid protein, which is expressed in all organs and distributed throughout the cytoplasm, membrane, nucleus and endoplasmic reticulum of the cell (38). Previous studies reported that MTDH was frequently over-expressed in breast (39), gastric (40), cervical (41) and bladder cancer (42). In glioma, MTDH was significantly upregulated at the mRNA and protein level. Additionally, the MTDH expression level was associated with the histological grade of patients with glioma (43). Experiments have demonstrated that MTDH acts as an oncogene and is associated with tumorigenesis and tumor development in glioma (22,44,45). Therefore, targeting MTDH in glioma may prolong survival time and improve the outcome of patients afflicted with this aggressive and invariably fatal disease. The present study suggested that miR-216b targeted MTDH to inhibit cell proliferation and invasion of glioma. Therefore, miR-216b may be useful as a therapeutic target for the suppression of the rapid growth and metastasis of gliomas.

In conclusion, the results of the present study indicated that miR-216b repressed cell proliferation and invasion through inhibiting the expression of MTDH, and highlight the therapeutic potential of miR-216b in glioma. Future work is required



to investigate the upstream regulatory mechanism of miR-216b in glioma and investigate whether the potential of miR-216b may be fully realised in patients with this malignancy.

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