

MicroRNA-559 plays an inhibitory role in the malignant progression of glioblastoma cells by directly targeting metadherin

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Purpose: Several microRNAs (miRNAs) that are aberrantly expressed in glioblastoma multiforme (GBM) play a significant role in GBM formation and progression. The expression profile and functions of miR-559 in GBM remain unclear. Here, we quantified the expression and investigated the involvement of miR-559 in the oncogenicity of GBM cells in vitro and in vivo.

Material and methods: Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was carried out to determine miR-559 expression in GBM tissues and cell lines. A series of functional assays was performed to evaluate the effects of miR-559 overexpression on GBM cell proliferation, apoptosis, migration, and invasion in vitro and on GBM tumor growth in vivo. The regulatory mechanisms of miR-559 action in GBM cells were then explored.

Results: The expression of miR-559 was lower in GBM tissues and cell lines and significantly correlated with the Karnofsky performance score and tumor size among patients with GBM. Exogenous miR-559 expression inhibited GBM cell proliferation, migration, and invasion and promoted apoptosis. MiR-559 overexpression decreased tumor growth in vivo. Mechanistic experiments confirmed metadherin (*MTDH*) as a direct target gene of miR-559 in GBM. Silencing of *MTDH* induced effects similar to those of miR-559 upregulation in GBM cells, whereas *MTDH* expression restoration attenuated the antitumor effects of miR-559 in GBM cells. Protein kinase B (AKT) in the phosphatase and tensin homolog (PTEN)-AKT signaling pathway was found to be deactivated in GBM cells after upregulation of miR-559 both in vitro and in vivo.

Conclusion: MiR-559 acts as a tumor suppressor in GBM cells in vitro and in vivo, at least in part through the downregulation of *MTDH* and inhibition of AKT in the PTEN-AKT pathway. Therefore, targeting the miR-559-*MTDH* axis may be a promising therapeutic strategy for patients with GBM.

Keywords: microRNA-559, glioblastoma, oncogenicity, metadherin, target therapy

Introduction

Glioma is the most common type of malignant primary brain tumor in adults¹ and can be categorized into four histopathological grades, namely, World Health Organization grades I-IV,² based on its malignant nature. Glioblastoma multiforme (GBM), a World Health Organization grade IV glioma, is the most common and aggressive histological subtype of glioma.³ The characteristics of GBM include rapid invasive growth, diffused infiltration, strong angiogenesis, and extensive

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genomic instability.⁴ At present, the primary therapeutic methods for patients with GBM include surgical resection, radiotherapy, and chemotherapy.⁵ Despite tremendous progress in the techniques for GBM diagnosis and therapy, the clinical outcomes of patients with GBM remain dissatisfactory, and the median survival time is less than 20 months after diagnosis.⁶ Therefore, elucidation of the molecular mechanisms underlying the initiation and progression of GBM may provide the theoretical basis for identification of effective therapeutic methods.

MicroRNAs (miRNAs) are a group of highly conserved single-stranded noncoding RNA molecules that are 17–24 nucleotides long.⁷ MiRNAs are implicated in gene regulation through the recognition and preferential binding to the 3' untranslated region (3'-UTRs) of their target mRNA(s), resulting in target mRNA cleavage or translation inhibition.⁸ MiRNA are frequently located in the genomic regions involved in cancer, indicating the critical role of miRNAs in carcinogenesis including cancer progression.⁹ MiRNAs are dysregulated in most of human cancers, including GBM,¹⁰ gastric cancer,¹¹ osteosarcoma,¹² tongue squamous cell carcinoma,¹³ and breast cancer.¹⁴ Some miRNAs are aberrantly expressed in GBM, including miR-143,¹⁵ miR-204,¹⁶ miR-574,¹⁷ and miR-744.¹⁸ Several lines of evidence have revealed that the aberrant expression of miRNA contributes to GBM initiation and progression through the regulation of aggressive cellular behavior.^{19–21} Thus, miRNAs are potential diagnostic and prognostic biomarkers and may serve as therapeutic targets in GBM.

The expression profile and functional characteristics of miR-559 in GBM remain unclear. Here we quantified the expression of miR-559 and investigated its role in the oncogenicity of GBM cells in vitro and in vivo. The molecular mechanisms underlying the antitumor actions of miR-559 in GBM cells were explored in detail. We demonstrate the inhibitory effects of miR-559 on the aggressive behavior of GBM in vitro and in vivo; this miRNA directly targets metadherin (*MTDH*) mRNA and inactivates protein kinase B (AKT) in the phosphatase and tensin homolog (PTEN)–AKT pathway.

Materials and methods

Patient samples

Forty-nine pairs of GBM samples and adjacent normal brain tissue samples were collected from the patients with GBM admitted to The Third People's Hospital of Linyi between June 2015 and November 2017. None of the enrolled patients

received chemotherapy or radiotherapy prior to surgical resection. After the operation, all tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA or protein isolation. The study protocol was approved by the ethics committee of The Third People's Hospital of Linyi and was conducted in accordance with the Declaration of Helsinki. Signed informed consent was provided by all the study participants.

Cell lines

Four GBM cell lines, U138, U251, LN228, and T98, were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (all from Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Normal human astrocytes (NHAs) were acquired from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained in the astrocyte medium (ScienCell Research Laboratories) containing 10% of FBS. All the cell lines were cultured at 37°C in a humidified atmosphere containing 5% of CO_2 .

Oligonucleotides, plasmids, and transfection

MiR-559 mimics and miRNA negative control mimics (miR-NC) were purchased from Guangzhou RiboBio Co., Ltd., (Guangzhou, China). Small-interfering RNA (siRNA) against MTDH (si-MTDH) and noncoding control siRNA (si-ctrl) were chemically synthesized by GenePharma (Shanghai, China). Upregulation of MTDH was achieved using the MTDH overexpression plasmid pcDNA3.1-MTDH (pc-MTDH; Chinese Academy of Sciences; Changchun, China). The empty pcDNA3.1 plasmid served as a control. For transfection, cells were seeded in six-well plates and cultured until 60–80% confluence. The cells were transfected with oligonucleotides or plasmids by means of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). Transfected cells were harvested 24, 48, or 72 h post-transfection for further experiments.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted with the TRIzol Reagent (Thermo Fisher Scientific). The One Step SYBR®

PrimeScript™ miRNA RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was utilized for the quantification of miR-559 expression. For the detection of *MTDH* mRNA expression, the complementary DNA (cDNA) was prepared from total RNA using the Prime-Script RT Reagent Kit (Takara Biotechnology Co., Ltd.). qPCR was carried out via the SYBR Premix Ex Taq™ Kit (Takara Biotechnology Co., Ltd.). U6 small nuclear RNA and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA served as endogenous controls for miR-559 and *MTDH*, respectively. All the reactions were conducted on an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific), and the data were analyzed by the $2^{-\Delta\Delta C_q}$ method.²²

Cell counting kit-8 (CCK-8) assay

Cellular proliferation was evaluated by the CCK-8 assay. In particular, transfected cells were seeded in 96-well plates at a density of 2.0×10^3 cells/well. The cells were cultured at 37 °C and 5% CO₂. The CCK-8 assay was performed at four time points (0, 24, 48, and 72 h) after seeding. At the indicated time points, 10 µL of the CCK8 solution (Dojindo, Kumamoto, Japan) was added into each well, followed by which cells were incubated at 37 °C for an additional 2 h. Absorbance was read at the 450 nm wavelength on a microtiter plate reader (Bio-Rad Instruments, Winooski, VT, USA).

Flow-cytometric analysis

Apoptosis was quantitated using the Annexin-V/fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BioLegend, San Diego, CA, USA). Transfected cells were harvested by means of 0.5% trypsin and washed with ice-cold phosphate buffered saline (PBS; Gibco; Thermo Fisher Scientific). The cells were resuspended in 100 µL of binding buffer, followed by double staining with 5 µL of the Annexin V-FITC conjugate and 5 µL of the propidium iodide solution. After 15 min incubation in the dark, the apoptosis rate was measured on a flow cytometer (FACScan™; BD Biosciences, Franklin Lakes, NJ, USA). All assays were performed in triplicate and repeated at least thrice.

In vitro migration and invasion assays

A total of 200 µL of a cell suspension containing 5×10^4 cells in FBS-free DMEM was seeded in the upper chamber of Transwell membrane inserts with 8 µm pore size (BD Biosciences). Approximately 500 µL of DMEM

supplemented with 10% of FBS was placed into the lower chambers. The filter membranes precoated with Matrigel (BD Biosciences) were employed for the in vitro invasion assay, whereas the in vitro migration assay was carried out with filter membranes not coated with Matrigel. After 24 h incubation, a cotton swab was used to gently remove the cells remaining on the upper side of the membrane surface. The cells that passed through membranes were fixed with 4% formaldehyde and stained with a 0.5% crystal violet solution. The numbers of migrated and invaded cells were determined in five randomly chosen visual fields under an Olympus inverted light microscope ($\times 200$ magnification; Olympus Corporation, Tokyo, Japan).

An in vivo xenograft model

Four-week-old BALB/c nude mice were purchased from Shanghai Laboratory Animal Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). MiR-559 mimic-transfected or miR-NC-transfected cells were subcutaneously injected into the flanks of the nude mice. A total of eight nude mice were used in this experiment and subdivided into two groups (n=4 for each group). One group was injected with miR-559 mimic-transfected cells, whereas miR-NC-transfected cells were injected in the other group. Once tumor xenografts became visible, their width (W) and length (L) were measured with a Vernier caliper every 7 days. The volume of tumor xenografts was calculated according to the formula $volume = 0.5 \times (L \times W^2)$. All nude mice were euthanized via fracture dislocation of the cervical spine, and the tumor xenografts were resected for total-RNA and total-protein isolation. The animal experimental procedures were approved by the Ethics Committee of The Third People's Hospital of Linyi. The animal experiments were conducted in accordance with the Declaration of Helsinki and the guidelines of The Third People's Hospital of Linyi.

Bioinformatic analysis and a luciferase reporter assay

Three well-established miRNA target prediction and functional study databases, TargetScan (http://www.targetscan.org/vert_72/), miRDB (<http://mirdb.org/>), and microRNA.org (<http://www.microrna.org/microrna/microrna/home.do>), were searched for the potential targets of miR-559.

The 3'-UTR fragment of human *MTDH* containing the predicted wild-type (wt) miR-559-binding site or its mutated (mut) version was amplified by Guangzhou

RiboBio Co., Ltd., and cloned into the psiCHECK-2 vector (Promega Corporation, Madison, WI, USA). Cells were seeded in 24-well plates at a density 10^5 cells/well. The generated luciferase plasmids were cotransfected with miR-559 mimics or miR-NC into the cells using Lipofectamine 2000. After 48 h, the transfected cells were harvested and subjected to detection of luciferase activities via a Dual-Luciferase Reporter assay system (Promega Corporation). *Renilla* luciferase activity was normalized to that of firefly luciferase activity.

Protein isolation and western blotting

Total protein was isolated from tissue samples, cells, or tumor xenografts using radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Proteins were denatured, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 10% gel, and transferred onto polyvinylidene fluoride membranes. After blockage with 5% fat-free dried milk diluted in Tris-buffered saline (TBS) containing 0.1% of Tween 20 for 2 h, the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-MTDH (cat. # ab126785; dilution 1:500; Abcam, Cambridge, MA, USA), anti-PTEN (ab32072; 1:500; Abcam), anti-phospho-(p-)AKT (ab181616; 1:500; Abcam), anti-AKT (ab155890; 1:500; Abcam), and anti-GAPDH (ab8227; 1:500; Abcam). A horseradish peroxidase-conjugated secondary antibody (dilution 1:3,000; cat. # 70758; Cell Signaling Technology, Inc., Danvers, MA, USA) was incubated with the membranes at room temperature for 2 h. The Enhanced Chemiluminescence (ECL) Western Blotting Kit (Pierce Biotechnology Inc., Rockford, IL, USA) was employed for visualization of the protein signals.

Statistical analysis

Each assay was repeated at least thrice, and the results were presented as the mean \pm standard deviation. The χ^2 test was carried out to assess the association between miR-559 expression and clinical parameters in patients with GBM. Student's *t*-test was conducted to assess a difference between two groups. Statistical significance of differences among at least three groups was evaluated by one-way analysis of variance followed by Tukey's *post hoc* test. Correlation between miR-559 and MTDH expression in GBM tissue samples was assessed by Spearman's correlation analysis. All statistical calculations were performed in the SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). A *P*-value <0.05 indicated a significant difference.

Results

MiR-559 expression is low in GBM tissues and cell lines

To determine the expression profile of miR-559 in GBM, we detected its expression in four GBM cell lines: U138, U251, LN229, and T98. NHAs served as a control. The data obtained from RT-qPCR analysis showed that the expression of miR-559 (varied levels) was lower in all four GBM cell lines than in NHAs (Figure 1A, $P<0.05$). Consistent with this result, miR-559 expression was found to be downregulated in GBM tissues as compared with the adjacent normal brain tissues (Figure 1B, $P<0.05$).

To test whether the miR-559 downregulation is related to GBM progression, 49 patients with GBM were subdivided into two groups, namely, the miR-559 low-expression group and miR-559 high-expression group, based on the median value of miR-559 expression among

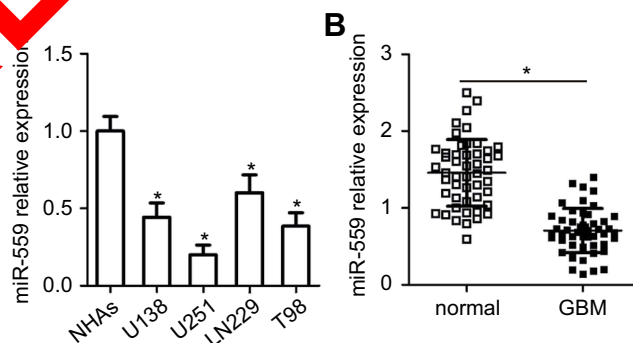


Figure 1 MiR-559 expression is low in GBM tissues and cell lines. (A) The expression of miR-559 in four GBM cell lines (U138, U251, LN229, and T98) and in NHAs was measured by RT-qPCR. * $P<0.05$ as compared with NHAs. (B) The expression of miR-559 in 49 pairs of GBM samples and adjacent normal brain tissues was assessed by RT-qPCR. * $P<0.05$ as compared with normal brain tissue samples.

Abbreviation: miR-559, microRNA-559; GBM, glioblastoma; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

the GBM tissue samples. Spearman's analysis revealed a correlation of low miR-559 expression with the Karnofsky performance score ($P=0.032$) and with tumor size ($P=0.004$) among the patients with GBM (Table 1). These results suggested that miR-559 expression is low in GBM, resulting in GBM progression.

MiR-559 upregulation inhibits GBM cell proliferation, migration, and invasion and promotes apoptosis in vitro

Cell lines U251 and T98 showed the lowest expression of miR-559 among the four tested GBM cell lines; therefore, these two GBM cell lines were selected for the subsequent experiments. To examine the role of miR-559 in GBM cells, miR-559 mimics were transfected into U251 and T98 cells; miR-NC served as a control. RT-qPCR analysis confirmed that miR-559 was overexpressed in U251 and T98 cells transfected with miR-559 mimics (Figure 2A, $P<0.05$). The CCK-8 assay was performed to determine the effect of miR-559 expression in GBM cells. Upregulation of miR-559 significantly decreased the proliferative ability of U251 and T98 cells as compared with the cells expressing miR-NC (Figure 2B, $P<0.05$). Because the suppression of cell proliferation is often linked with apoptosis, we determined

whether miR-559 overexpression affects the apoptosis of GBM cells. The results of flow-cytometric analysis revealed that the transfection of miR-559 mimics into U251 and T98 cells increased apoptosis as compared with miR-NC transfection (Figure 2C, $P<0.05$). Furthermore, in vitro migration and invasion assays were conducted to determine the participation of miR-559 in the metastasis of GBM cells. The ectopic miR-559 expression noticeably decreased the migration (Figure 2D, $P<0.05$) and invasion (Figure 2E, $P<0.05$) abilities of U251 and T98 cells. Therefore, miR-559 can inhibit the growth and metastasis of GBM cells in vitro.

MTDH mRNA is a direct target of miR-559, and MTDH expression inversely correlates with miR-559 levels in clinical GBM samples

To reveal the mechanisms underlying miR-559-mediated inhibition of the malignant progression of GBM, we performed bioinformatic analysis and identified putative miR-559 targets. *MTDH*, a well-known oncogene, contains a 3'-UTR sequence complementary to the seed sequences of miR-559 (Figure 3A). To verify this prediction, the luciferase reporter assay was conducted to evaluate the possibility of the direct binding of miR-559 to the 3'-UTR of *MTDH* mRNA. As expected, miR-559 overexpression substantially decreased the luciferase activity of the plasmid containing wild-type *MTDH* 3'-UTR in both U251 and T98 cells ($P<0.05$); however, the luciferase activity of the plasmid harboring the mutated *MTDH* 3'-UTR was barely affected (Figure 3B).

To test whether miR-559 modulates MTDH expression, we quantified MTDH mRNA and protein expression in U251 and T98 cells after transfection with miR-559 mimics or miR-NC. As evidenced by RT-qPCR and western blot analyses, MTDH expression in U251 and T98 cells dramatically decreased at both mRNA (Figure 3C, $P<0.05$) and protein levels (Figure 3D, $P<0.05$) in response to the upregulation of miR-559. To further clarify the relation between miR-559 and MTDH expression, we measured MTDH expression in GBM samples and adjacent normal brain tissue samples by RT-qPCR. The levels of MTDH mRNA (Figure 3E, $P<0.05$) and protein (Figure 3F, $P<0.05$) were higher in GBM samples than in the adjacent normal brain tissue samples. Spearman's analysis confirmed the negative correlation between miR-559 and *MTDH* mRNA levels in GBM tissue samples (Figure 3G; $R^2=0.3215$, $P<0.0001$). Therefore, these results indicate that MTDH mRNA serves as a direct target of miR-559 in GBM.

Table 1 Correlation of miR-559 expression with GBM clinical pathological characteristics

Characteristics	miR-559 expression		p
	Low	High	
Gender			0.483
Male	17	14	
Female	10	10	
Age			0.176
<50 years	13	7	
≥50 years	13	17	
Extension resection			0.628
Subtotal	10	8	
Total	15	16	
KPS			0.032*
≥80	7	14	
<80	18	10	
Tumor size			0.004*
<5 cm	11	20	
≥5 cm	14	4	

Note: * $P<0.05$.

Abbreviation: KPS, Karnofsky performance score.

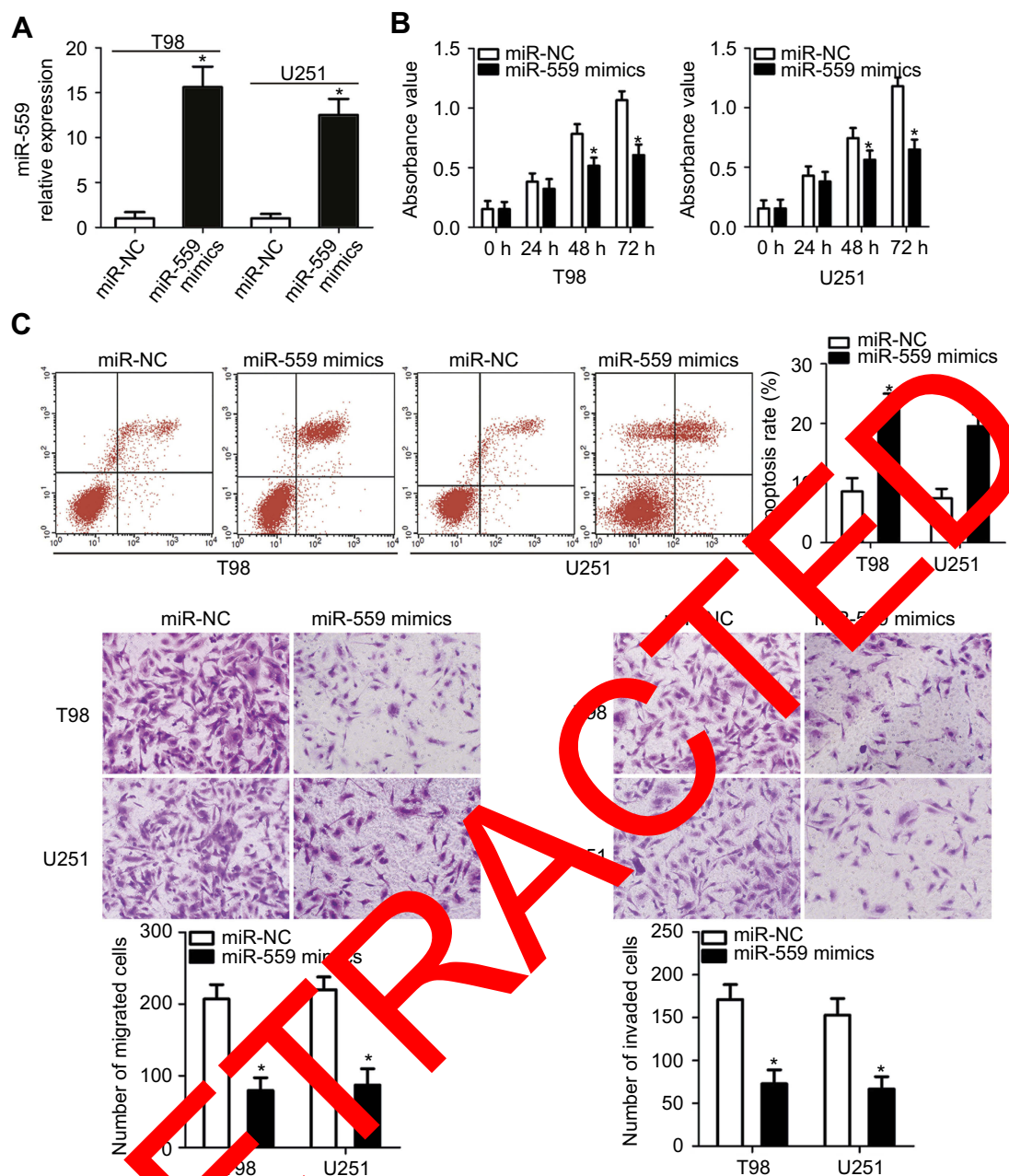


Figure 2 Exogenous miR-559 expression inhibits proliferation, migration, and invasiveness and promotes apoptosis of U251 and T98 cells. **(A)** RT-qPCR was carried out to measure miR-559 expression in U251 and T98 cells after transfection of miR-559 mimics or miR-NC. * $P < 0.05$ as compared with group miR-NC. **(B)** U251 and T98 cells were transfected with miR-559 mimics or miR-NC. The transfected cells were subjected to the CCK-8 assay to evaluate cellular proliferation at every time point. * $P < 0.05$ as compared with group miR-NC. **(C)** Flow cytometric analysis was conducted to quantitate the apoptosis of U251 and T98 cells that were transfected with miR-559 mimics or miR-NC. The Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BioLegend) was used in the flow-cytometric analysis. * $P < 0.05$ as compared with group miR-NC. **(D, E)** The effects of miR-559 upregulation on U251 and T98 cell migration and invasion were evaluated by in vitro migration and invasion assays ($\times 200$ magnification). * $P < 0.05$ as compared with group miR-NC.

Abbreviation: miR-559, microRNA-559; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; miR-NC, miRNA negative control mimics; CCK-8, Cell counting kit-8 (CCK-8) assay.

Silencing of MTDH expression elicits effects similar to those of miR-559 mimics in GBM cells

After identifying *MTDH* mRNA as a direct target of miR-559 in GBM cells, we determined whether MTDH acts as a downstream effector of miR-559. MTDH was knocked

down in U251 and T98 cells through the transfection of si-MTDH (Figure 4A, $P < 0.05$). The effects of MTDH down-regulation on cellular proliferation and apoptosis were investigated in U251 and T98 cells by the CCK-8 assay and flow-cytometric analysis. Inhibition of MTDH expression suppressed the proliferation (Figure 4B, $P < 0.05$) and

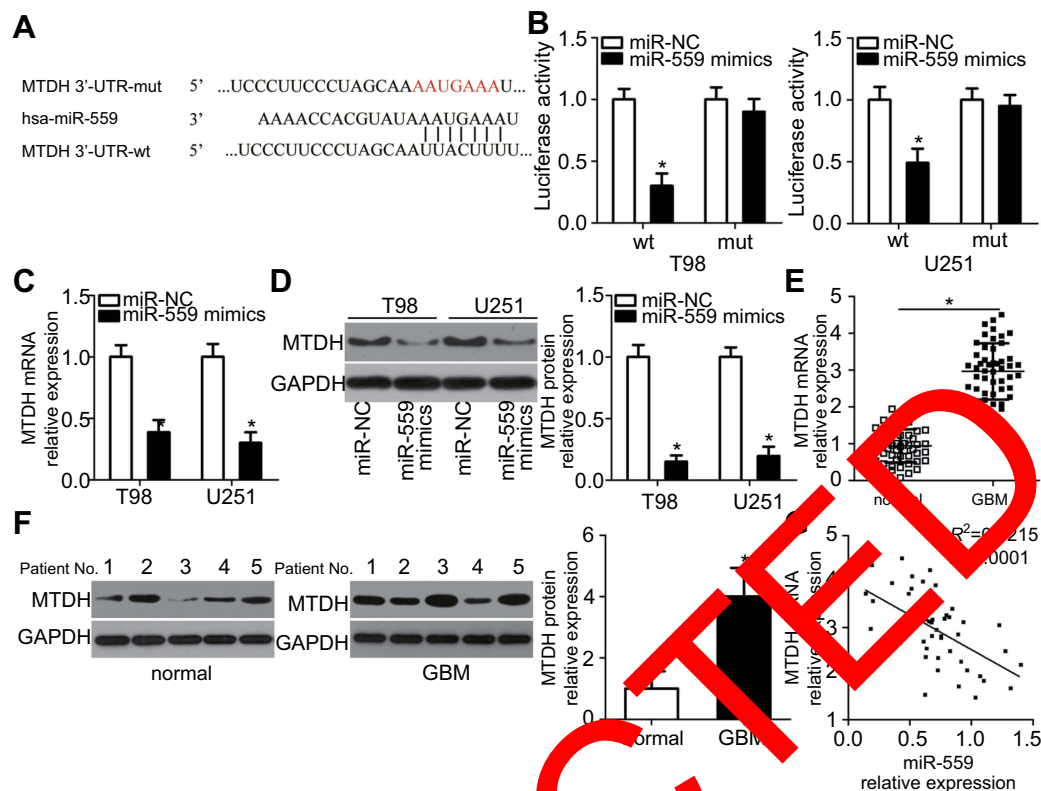


Figure 3 MTDH is a direct target gene of miR-559 in GBM cells. (A) Illustration of the wild-type and mutated binding sites of miR-559 in the 3'-UTR of MTDH mRNA. (B) A luciferase reporter assay was conducted to detect the luciferase activity of U251 and T98 cells that were cotransfected with miR-559 mimics or miR-NC and either the wild-type or mutated luciferase reporter plasmid. * $P<0.05$ as compared with group miR-NC. (C, D) Expression levels of MTDH mRNA and protein in miR-559-overexpressing U251 and T98 cells were detected by RT-qPCR and western blotting, respectively. * $P<0.05$ as compared with group miR-NC. (E, F) The expression levels of MTDH mRNA and protein were analyzed in GBM samples and adjacent normal brain tissue through RT-qPCR and western blotting, respectively. * $P<0.05$ as compared with normal brain tissue samples. (G) Spearman's correlation analysis of miR-559 and MTDH mRNA levels in human GBM specimens. $R^2=0.3215$, $P<0.0001$. **Abbreviation:** miR-559, microRNA-559; RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR-NC, miRNA negative control mimics.

increased the apoptosis (Figure 3C, $P<0.05$) of U251 and T98 cells. In vitro migration and invasion assay indicated a decrease in the migratory (Figure 4D, $P<0.05$) and invasive abilities of U251 and T98 cells (Figure 4E, $P<0.05$) after the MTDH knockdown as compared with the control cells.

MTDH expression restoration reverses the miR-559-induced tumor-suppressive effects on GBM cell behavior

Rescue experiments were conducted to test whether the effects of miR-559 overexpression on GBM cell behavior were mediated by the downregulation of MTDH. Because MTDH was found to be downregulated in U251 and T98 cells transfected with miR-559 mimics, we restored MTDH expression in miR-559-overexpressing cells through the transfection of pcDNA3.1-MTDH (Figure 5A, $P<0.05$), and a series of functional experiments was carried out.

The CCK-8 assay indicated that the restoration of MTDH expression attenuated the suppressive effects of miR-559 on U251 and T98 cell proliferation (Figure 5B, $P<0.05$). In agreement with these results, MTDH expression restoration reduced the effects of miR-559 overexpression on apoptosis (Figure 5C, $P<0.05$), migration (Figure 5D, $P<0.05$), and invasiveness (Figure 5E, $P<0.05$) of U251 and T98 cells. Taken together, these observations suggested that MTDH acts as a downstream mediator of miR-559 in the regulation of GBM cell behavior.

MiR-559 inhibits AKT in the PTEN-AKT pathway in GBM cells

Some studies have uncovered the involvement of MTDH in the regulation of the PTEN-AKT signaling pathway.^{23,24} We determined whether miR-559 inhibits the output of the PTEN-AKT pathway in GBM cells. MiR-559 mimics and pcDNA3.1 or pcDNA3.1-MTDH

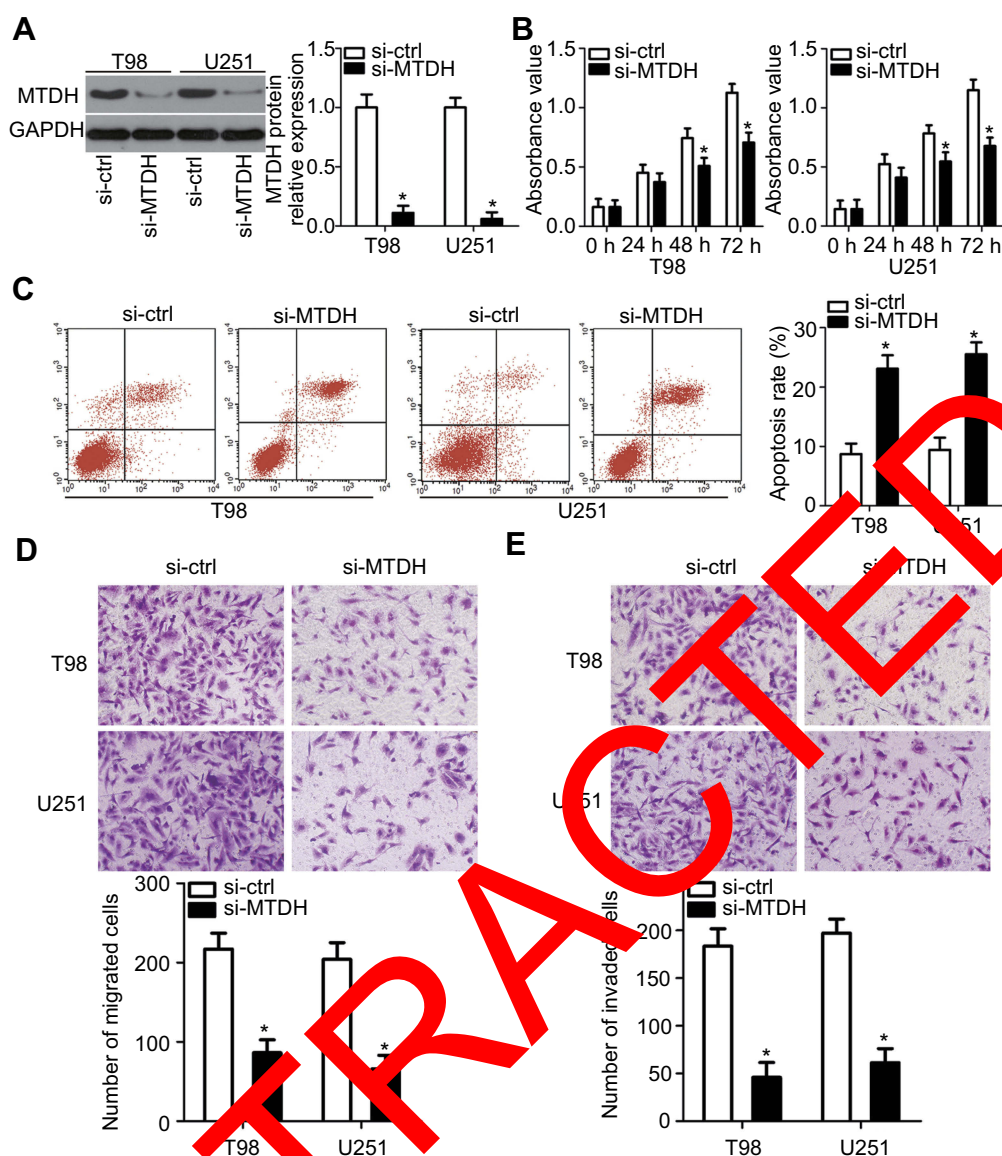


Figure 4 The MTDH knockdown in U251 and T98 cells has effects similar to those observed with miR-559 overexpression. U251 and T98 cells were transfected with si-MTDH or si-ctrl and were studied in the following experiments: **(A)** After 72 h of transfection, western blot analysis was carried out to assess MTDH protein expression. * $P < 0.05$ as compared with group si-ctrl. **(B)** The proliferation and apoptosis of U251 and T98 cells after the MTDH knockdown were determined with the CCK-8 assay and flow-cytometric analysis. * $P < 0.05$ as compared with group si-ctrl. **(D, E)** In vitro migration and invasion assays were performed to evaluate the migratory and invasive abilities of U251 and T98 cells after transfection with si-MTDH or si-ctrl ($\times 200$ magnification). * $P < 0.05$ as compared with group si-ctrl.

were introduced into U251 and T98 cells, and western blot analysis was performed to measure MTDH, p-AKT, and AKT protein amounts. In comparison with the cells transfected with miR-NC, those transfected with miR-559 mimics contained larger PTEN and lower p-AKT protein amounts (Figure 6). The restored MTDH expression attenuated the change in PTEN and p-AKT amounts induced by miR-559 overexpression (Figure 6). These results suggested that miR-559 deactivates AKT in the PTEN-AKT signaling pathway in GBM cells via the negative regulation of MTDH expression.

Overexpression of mir-559 hinders tumor growth in vivo via inhibition of the MTDH-PTEN-AKT pathway

We set up an in vivo xenograft model to determine the effect of miR-559 expression on tumor growth in vivo. Representative images of the tumor xenografts derived from miR-559-transfected or miR-NC-transfected U251 cells are shown in Figure 7A. The tumor volume in the mice from the miR-559 mimic group was much smaller than that in the miR-NC group (Figure 7B, $P < 0.05$).

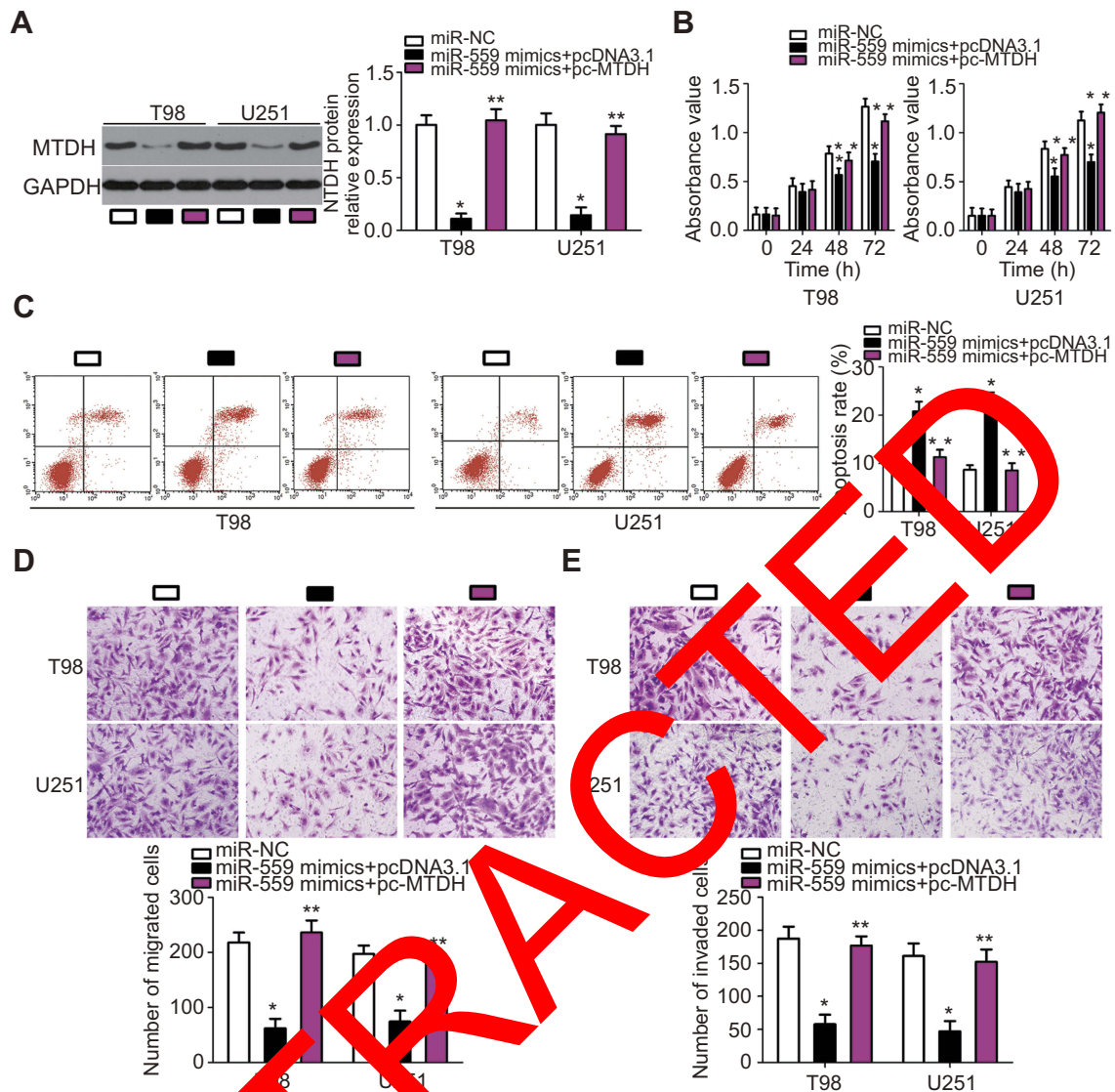


Figure 5 MTDH reintroduction reverses the anti-tumor effects of miR-559 in GBM cells. Rescue experiments were carried out by introducing pcDNA3.1-MTDH or pcDNA3.1 along with miR-559 mimics into U251 and T98 cells. (A) The protein level of MTDH in these cells was measured by western blotting. * $P < 0.05$ as compared with group miR-NC. ** $P < 0.05$ in comparison with group miR-559 mimics+pcDNA3.1. (B–E) The proliferation, apoptosis, migration, and invasiveness of the aforementioned cells were investigated via the CCK-8 assay, flow cytometry, and in vitro migration and invasion assays ($\times 200$ magnification), respectively. * $P < 0.05$ as compared with group miR-NC. ** $P < 0.05$ relative to group miR-559 mimics+pcDNA3.1.

Abbreviation: miR-559, miR-559; miR-NC, miRNA negative control mimics; CCK-8, Cell counting kit-8 (CCK-8) assay

Besides, the weight of tumor xenografts was smaller in the miR-559 mimic group than in the miR-NC group (Figure 7C, $P < 0.05$). Western blot analysis uncovered downregulation of MTDH and p-AKT and elevation of PTEN expression in the tumor xenografts from the miR-559 mimic group (Figure 7D). The tumor xenografts were analyzed for miR-559 expression by RT-qPCR. The data confirmed the higher expression of miR-559 in the tumor xenografts derived from the miR-559 mimic group in comparison with the control group (Figure 7E, $P < 0.05$). Thus, miR-559 decreased the growth of the GBM tumor in vivo via repression of the MTDH–PTEN–AKT pathway.

Discussion

Abnormal expression of miRNAs is a common feature of human malignant tumors, including GBM.²⁵ Expression of several miRNAs has been reported to be dysregulated in GBM and to play a significant part in GBM formation and progression.^{26–28} MiRNAs may perform tumor-suppressive or tumor-promoting functions in GBM through regulation of the expression of their target genes.²⁹ Hence, elucidation of the functions of cancer-associated miRNAs in GBM may facilitate the identification of promising therapeutic targets in GBM. Herein, we studied the miR-559 expression in GBM and investigated

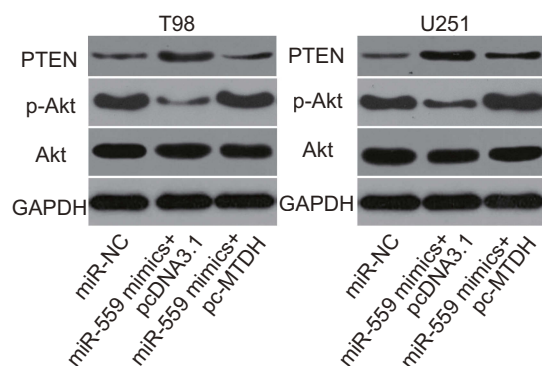


Figure 6 MiR-559 targets (and downregulates) MTDH to inactivate the MTDH–PTEN–AKT pathway in U251 and T98 cells. MiR-559 mimics along with pcDNA3.1-MTDH or pcDNA3.1 were introduced into U251 and T98 cells. After 72 h, western blotting was conducted to assess PTEN, p-AKT, and AKT protein levels.

Abbreviation: miR-559, microRNA-559;

its clinical significance. In addition, we determined the specific roles of miR-559 in the oncogenicity of GBM in vitro and in vivo. The mechanisms responsible for the activity of miR-559 in GBM cells were explored in detail.

Although thousands of miRNAs have been identified, studies on the expression profile and functional characterization of miR-559 in GBM are limited. Herein, we

performed RT-qPCR to measure miR-559 expression in GBM tissues and cell lines. The expression of miR-559 was obviously lower in GBM tissue samples and cell lines than in adjacent normal brain tissues and NHAs, respectively. The decreased miR-559 expression notably correlated with the Karnofsky performance score and tumor size among patients with GBM. Exogenous miR-559 expression suppressed cell proliferation, migration, and invasiveness and increased apoptosis of GBM cells. In addition, the upregulation of miR-559 reduced GBM tumor growth in vivo. These results suggest that miR-559 has an inhibitory effect on the oncogenic properties and malignant progression of GBM cells; thus, miR-559 may serve as a potential therapeutic agent and as a biomarker for the diagnosis of GBM.

An important characteristic of miRNAs is their direct binding to the 3'UTRs of their target mRNAs, thereby resulting in target mRNA degradation or translation inhibition. Accordingly, we carried out miRNA target prediction and functional study in databases TargetScan, miRDB, and microRNA.org. The bioinformatic prediction showed that the 3'-UTR of *MTDH* mRNA contains a highly conserved binding site for miR-559. In addition,

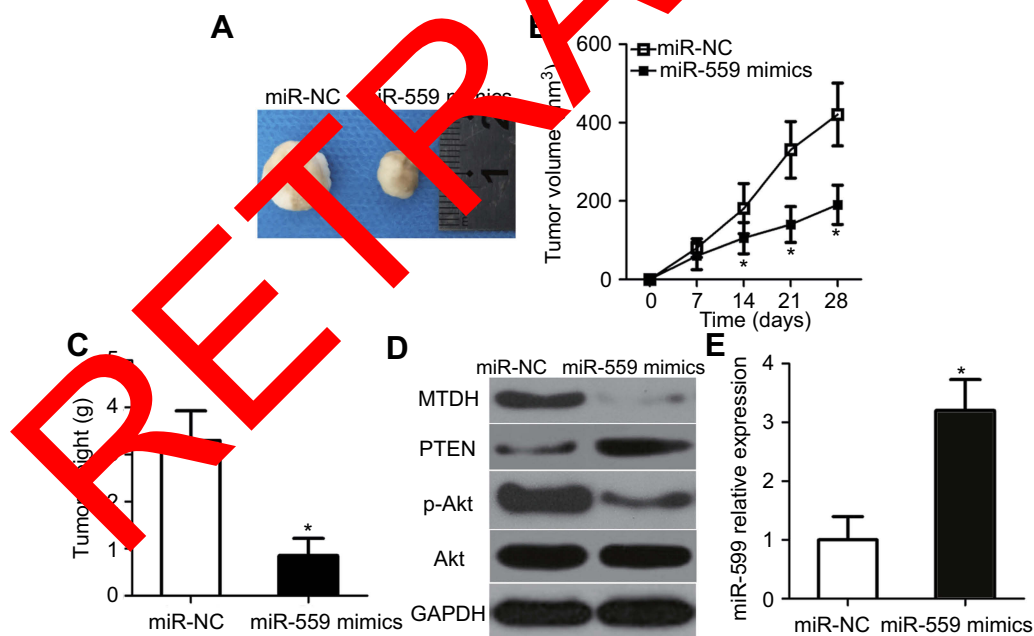


Figure 7 MiR-559 overexpression suppresses GBM tumor growth in vivo. **(A)** Representative images of the tumor xenografts 4 weeks after inoculation with U251 cells expressing miR-559 mimics or miR-NC. **(B)** Tumor growth curves after the subcutaneous injection of nude mice with U251 cells transfected with miR-559 mimics or miR-NC. The volume of tumor xenografts was measured every 7 days. * $P < 0.05$ as compared with group miR-NC. **(C)** Changes in the weight of tumor xenografts derived from miR-559 mimic-transfected or miR-NC-transfected U251 cells. * $P < 0.05$ relative to group miR-NC. **(D)** Western blotting was conducted to measure MTDH, PTEN, p-AKT, and AKT protein amounts in the tumor xenografts. **(E)** The upregulation of miR-559 in tumor xenografts from the miR-559 mimic group was confirmed by RT-qPCR. * $P < 0.05$ in comparison with group miR-NC.

Abbreviation: miR-559, microRNA-559; GBM, glioblastoma; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; miR-NC, miRNA negative control mimics.

the luciferase reporter assay revealed that miR-559 directly binds to the 3'-UTR of *MTDH* mRNA in GBM cells. Furthermore, *MTDH* mRNA and protein expression in GBM cells decreased after miR-559 overexpression. *MTDH* turned out to be notably upregulated in GBM tissues and negatively correlated with miR-559 levels. In addition, the knockdown of *MTDH* simulated the effects of miR-559 upregulation in GBM cells, whereas the recovery of *MTDH* expression attenuated the miR-559-mediated anticancer effects in GBM cells. Thus, *MTDH* acts as an immediate downstream mediator of miR-559 in GBM cells. Validation of the direct target of miR-559 in GBM is necessary not only to elucidate its functions in GBM but also for the identification of effective therapeutic methods for patients with this deadly disease.

MTDH, located in chromosomal region 8q22, is overexpressed in multiple types of human cancers, including non-small cell lung cancer,³⁰ thyroid carcinoma,³¹ hepatocellular carcinoma,³² and laryngeal squamous cell carcinoma.³³ *MTDH* has been implicated in the tumorigenesis including tumor progression because *MTDH* regulates several biological phenomena, including cellular proliferation, cell cycle, apoptosis, metastasis, epithelial-to-mesenchymal transition, and chemoradiotherapy sensitivity.^{34–36} *MTDH* expression is excessive in GBM and correlates with histological grade.³⁷ Patients with GBM overexpressing *MTDH* have a shorter survival period than do patients with low *MTDH* expression in the tumor. Overexpression of *MTDH* serves as an independent prognostic biomarker of GBM.³⁸ *MTDH* is known to promote the malignant progression of GBM cells.^{39–42} In the present study, we demonstrated that miR-559 represses the aggressive behavior of GBM cells in vitro and in vivo by directly targeting (and downregulating) *MTDH* and by reducing the output of the PTEN–AKT pathway. Hence, the suppression of *MTDH* expression by a miR-559-based targeted therapy may serve as an effective strategy for patients with GBM.

Conclusions

Our study indicates that miR-559 is a pleiotropic tumor-suppressive miRNA that directly targets *MTDH* mRNA and then suppresses the output of the PTEN–AKT pathway to exert antitumor action in GBM cells through the inhibition of cell growth and metastasis in vitro and a reduction of tumor growth in vivo. These results suggest that miR-559 may be an effective therapeutic candidate for

reducing or stopping the progression of GBM and may prevent its metastasis.

Abbreviation list

3'-UTR, 3' untranslated region; AKT, protein kinase B; GBM, glioblastoma multiforme; miRNA, microRNA; *MTDH*, metadherin; PTEN, phosphatase and tensin homolog; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; siRNA, small interfering RNA.

Disclosure

The authors report no conflict of interest in this work.

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