miR-216b inhibits glioma cell migration and invasion through suppression of FoxM1

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Abstract. MicroRNAs (miRNAs) play a vital role in tumour biological and pathologic processes. In the present study, we aimed to detect the expression and biological role of miR-216b in glioma. Our data showed that miR-216b was significantly downregulated in human glioma tissues and cells. Ectopic expression of miR-216b inhibited the proliferation and invasion of U87 and U251 cells and suppressed the growth of xenograft tumours in vivo. Bioinformatic and luciferase reporter analyses identified Forkhead box protein M1 (FoxM1) as a direct target of miR-216b. Overexpression of miR-216b inhibited the expression of FoxM1 in glioma cells. Rescue experiments demonstrated that co-transfection of FoxM1 lacking the 3'-untranslated region partially prevented miR-216b-induced inhibition of glioma cell growth and invasion. In vivo studies indicated that ectopic expression of miR-216b impeded the proliferation of glioma xenograft tumours in nude mice, coupled with a decreased in FoxM1 protein expression and the percentage of Ki-67-positive tumour cells. Taken together, our results provide evidence of the suppressive activity of miR-216b in glioma, which is largely ascribed to downregulation of FoxM1. Restoration of miR-216b may provide a novel potential therapeutic agent for glioma.

Introduction

Glioblastoma (GBM) is one of the most common and aggressive forms of primary brain tumour, with a median patient survival of 9-12 months. Despite therapeutic advances and new biological insights, ~10,000 new patients with high-grade or malignant glioma suffer from tumour recurrence each year (1,2). The lethality and poor prognosis of the disease are related to the highly invasive/migratory capacity of glioma cells, making complete surgical resection impossible (3,4). Therefore, it is essential to investigate novel and effective therapeutic approaches for glioma.

MicroRNAs (miRNAs) are a class of small non-coding RNAs which regulate gene expression post-transcriptionally causing target mRNA deadenylation and degradation in diverse biological processes, such as proliferation, differentiation, invasion and apoptosis (5). There is evidence suggesting that various miRNAs can potentially regulate hundreds of mRNAs and indicated that miRNAs are involved in tumourigenic processes (6). It has been reported that miRNAs are involved in the regulation of the expression of 1/3 of human protein-coding genes, accounting for ~1% of all the expressed human genes (7,8). Recent studies have shown that a number of miRNAs may function as oncogenes or tumour suppressor genes in glioma, including miR-21, miR-221/222, miR-124 and miR-27b (9-12). miR-216b has been demonstrated to function as a regulator in hepatocellular carcinoma (13). However, the function of miR-216b in gliomas remains unclear.

Forkhead box protein M1 (FoxM1, previously known as HFH-11, INS-1, WIN, MPP2/MPHOSPH2 or Trident/FKHL16) plays a vital role in animal development (14). Overexpression of FoxM1 is common in many types of cancers including glioma (15), and is involved in cell cycle progression, cell differentiation, DNA damage repair, angiogenesis and invasion. Recently, there is evidence showing that FoxM1 is a key regulator in cancer drug sensitivity and resistance (16). The Ki-67 protein (also known as MKi-67) is a marker of cell proliferation (17). In addition, it is associated with ribosomal RNA transcription. MMP-2 and MMP-9 are members of the matrix metalloproteinase (MMP) family. The major function of MMPs in cancer progression is their role in ECM degradation, which allows cancer cells to migrate out of the primary tumour to form metastases (18).

In high-grade glioma tissues and cells, miR-216b was found to be downregulated compared to normal brain tissues and normal human astrocyte (NHA) cells. In the present study, we found that FoxM1 is a direct target of miR-216b. Transient transfection of miR-216b mimics into glioma cell line suppressed cell proliferation and invasion. Overexpression of FoxM1 partially impeded the effects of miR-216b on the glioma biological behaviours. An in vivo study demonstrated...
that ectopic expression of miR-216b retarded the proliferation of U87 xenograft tumours in BALB/c mice. In addition, the percentage of Ki-67-positive tumour cells and FoxM1 protein expression had a significant decline compared to the control tumours. These results indicate that miR-216b may play a critical role in the regulation of the proliferation and invasion of glioma cells, suggesting that miR-216b could be a promising therapeutic target for glioblastoma (GBM).

Materials and methods

Cells and tissues. Human glioma cell lines (U87, T98G, LN229 and U251) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NHA cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). All cells were maintained in a 37°C/5% carbon dioxide incubator in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% foetal-bovine serum (FBS) (Gibco, Los Angeles, CA, USA).

Human GBM and adjacent normal brain tissues were collected from 36 patients with histologically confirmed GBM who underwent tumour resection at Xiangya Hospital of Central South University between May 2010 and December 2014. No patients had received any anticancer treatment. All tissues were pathologically confirmed and immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Written informed consent for research purposes was obtained from each patient. All procedures were subjected to the Declaration of Helsinki. In addition, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The present study was approved by the Ethical Committee of Central South University (Changsha, China).

Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RT-qPCR chain reactions were performed in triplicate in an ABI 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocols. The miR-216b levels were detected using the TaqMan MicroRNA Assay kit, and endogenous mRNA levels of FoxM1 were measured using SYBR-Green PCR Master Mix kit (Applied biosystems). The cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 15 sec and 60°C for 40 sec. U6 was used as an internal control. All assays were repeated as independent experiments at least 3 times. The optical density was detected at the wavelength of 490 nm. The data are presented as the mean ± standard error of the mean. All assays were repeated as independent experiments at least 3 times. Cells were plated in 6-well plates (300 cells/well) and incubated for 2 weeks. Then, cells were fixed with methanol and stained with violet (Sigma, St. Louis, MO, USA). The colonies (>50 cells) were counted.

Wound healing and Transwell assays. U87 and U251 cells were plated into 6-well plates. When the cell confluence reached ~90%, ~24 h after transfection, a scratch was made using a sterile tip on the monolayer. After wounding, debris was removed by washing cells with phosphate-buffered saline (PBS). Images of the scratched area were captured at 0 h and after 48 h at a magnification of x200 under a light microscope. The number of cells that had migrated into the wounded area or cells with extended protrusions from the wound border were counted and averaged. Experiments were repeated 3 times.

The Transwell filters (Costar, Cambridge, MA, USA) coated with Matrigel (Becton-Dickinson, Franklin Lakes, NJ, USA) were used to quantify in vitro glioma cell invasion. Transfected cells were cultured at 5×10^4/well into the upper compartment of the chamber in serum-free medium. Medium containing 20% FBS was added to the lower chamber. After 24 h of incubation at 37°C with 5% carbon dioxide, the medium was removed from the upper chamber. The non-invading cells were scraped off with a cotton swab while the bottom cells were fixed with 3% paraformaldehyde, stained with 0.1% Plasmids, oligonucleotides and cell transfection. miR-216b mimics and negative control (NC) oligonucleotides were purchased from GenePharma (Shanghai, China), miR-216b mimics or NC were obtained from GeneChem (Shanghai, China). Cells were transfected with miR-216b mimics, FoxM1 siRNA and NC (200 pmol each) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The FoxM1 open reading frame without the 3’UTR region was amplified by PCR with human FoxM1 cDNA as a template and inserted into the pcDNA3.1(+)(+)+ expression vector (Invitrogen). The human FoxM1 3’UTR-Luc reporter was created by the ligation of FoxM1 3’UTR PCR product into the XbaI site of the pcDNA3.1-control vector (Promega, Madison, WI, USA), to generate the plasmid pcDNA3.1-WT-FoxM1-3’UTR (FoxM1-wild). The mutant reporter was generated from pcDNA3.1-WT-FoxM1 3’UTR-Luc by replacing the binding site of miR-216b with a restriction enzyme cutting site GGUGACUC.

Luciferase reporter assay. For the luciferase reporter assay, U87 and U251 cells were co-transfected with luciferase reporter vectors and miR-216b using Lipofectamine 2000. Cells were transfected with pRL-TK (Promega). The Renilla luciferase activity was utilized as an internal control. Luciferase activity was analysed 48 h after transfection with a Dual-Luciferase Reporter Assay System (Promega).

Cell proliferation and colony formation assays. GBM cells were seeded into 96-well plates at 2,000 cells/well. Following transfection with miR-216b mimics, the MTT assay was used to measure the cell viability of human glioma cells, as previously described (9). Each experiment was performed in triplicate. The optical density was detected at the wavelength of 490 nm. The data are presented as the mean ± standard error of the mean. All assays were repeated as independent experiments at least 3 times. Cells were plated in 6-well plates (300 cells/well) and incubated for 2 weeks. Then, cells were fixed with methanol and stained with violet (Sigma, St. Louis, MO, USA). The colonies (>50 cells) were counted.

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Western blotting. Glioma tissues and cells were scraped in Thermo Scientific RIPA buffer (Pierce, Rockford, IL, USA) with protease inhibitors. The Pierce BCA protein assay kit was used to determine the protein concentration. The membranes were blocked with 5% non-fat dry milk (w/v) at room temperature for 1 h and incubated separately with rabbit anti-human FoxM1 (1:1,000), mouse anti-human MMP-2, MMP-9 (1:1,000), rabbit anti-human Ki-67 (1:1,000) (all from Cell Signaling Technology, Danvers, MA, USA) and mouse anti-human β-actin (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; ab150077; Abcam, Cambridge, MA, USA) for 1 h. The relative protein expression levels on the polyvinylidene fluoride (PVDF) membrane were scanned with the enhanced chemiluminescence (ECL) system and quantified using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA).

Tumour xenograft model and Ki-67 immunohistochemical staining. miR-216b-overexpressing and control U87 glioma cells (5 × 10^5 cells/mouse in 3 μl, pretreated with miR-216b and NC mimics) were injected into the intracranial space of 5-week-old female nude mice (n=14; Cancer Institute of the Chinese Academy of Medical Science) using a stereotactic instrument. Bioluminescence imaging was used to measure intracranial tumour growth. The mice were anesthetized, injected intraperitoneally with D-luciferin at 50 mg/ml and imaged with Bruker In-Vivo FX PRO Imaging System. At the end of the experiment (40 days after injection), mice were sacrificed and fixed with 4% paraformaldehyde. Tumours were processed for immunohistochemistry.

Paraffin-embedded tissue sections (4 μm) were deparaffinized and subjected to heat-mediated antigen retrieval. Sections were incubated with 3% H₂O₂ to quench endogenous peroxidase activity. After washing, sections were incubated with rabbit anti-Ki-67 and FoxM1 antibody (1:200; Cell Signaling Technology). Then, the sections were incubated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). After washing, the sections were developed with a Vectastain AbC (avidin-biotin complex) peroxidase kit (Vector Laboratories) and a 3,3′-diaminobenzidine (DAB) substrate (Sigma). The section was then counterstained with haematoxylin and the percentage of Ki-67- and FoxM1-positive cells were estimated under a microscope.

Statistical analysis. The data are expressed as the mean ± SD of 3 independent experiments. Statistics were estimated with Student's t-test. All differences were considered to be statistically significant at the level of P<0.05. Differences between groups were analysed using a one-way ANOVA or χ² test. The results were analysed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistics were performed using the SPSS Graduate Pack, version 17.0, statistical software (SPSS, Inc., Chicago, IL, USA).

Table I. Clinical characteristics of the glioma patients according to miR-216b level in tissue.

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of cases</th>
<th>miR-216b expression</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>19</td>
<td>Low 10 High 9</td>
<td>0.127</td>
</tr>
<tr>
<td>≥60</td>
<td>17</td>
<td>Low 13 High 4</td>
<td></td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>Low 15 High 7</td>
<td>0.374</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>Low 8 High 6</td>
<td></td>
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<td>KPS</td>
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<tr>
<td>≤5</td>
<td>25</td>
<td>Low 19 High 6</td>
<td>0.029¹</td>
</tr>
<tr>
<td>≥5</td>
<td>11</td>
<td>Low 4 High 7</td>
<td></td>
</tr>
<tr>
<td>Mean tumor diameter (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>15</td>
<td>Low 16 High 9</td>
<td>0.015¹</td>
</tr>
<tr>
<td>≥5</td>
<td>21</td>
<td>Low 17 High 4</td>
<td></td>
</tr>
<tr>
<td>Necrosis on MRI</td>
<td></td>
<td></td>
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<td>Low 11 High 10</td>
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<td>Low 12 High 3</td>
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<td>Low 9</td>
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</tr>
<tr>
<td>No</td>
<td>24</td>
<td>Low 14 High 10</td>
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</table>

¹χ² test. P-values in bold print indicate statistically significant differences. KPS, Karnofsky performance status; MRI, magnetic resonance imaging.

Results

miR-216b was downregulated in glioma tissues and cell lines. The expression of miR-216b in glioma and normal brain tissues was examined using RT-qPCR analysis. As shown in Fig. 1A, glioma tissues exhibited a significantly lower level of miR-216b compared to that noted in the adjacent normal brain tissues (P<0.001), miR-216b was consistently observed to decrease in the GBM cell lines when compared with the level noted in the NHA cell line (Fig. 1B). In addition, low expression of miR-216b was significantly associated with KPS score and mean tumour diameter (Table I) (P<0.05).

miR-216b inhibits glioma cell proliferation, migration and invasion in glioma cells. To determine the biological function of miR-216b downregulation in glioma, glioma cells were transfected with the miR-216b plasmid and evaluated for proliferation and invasion using the MTT, colony formation and Transwell assays. miR-216b expression was confirmed by RT-qPCR in the U87 and U251 cells (Fig. 2A). The result showed that miR-216b plays a vital role in suppressing the proliferation of glioma cells (Fig. 2B and C).

Cell invasion was analysed with the Transwell assays. After treatment with miR-216b, the number of invasive cells...
Figure 1. Expression of miR-216b is downregulated in glioma tissues and cell lines. (A) miR-216b levels in glioma patient samples and adjacent normal brain tissues were detected by RT-qPCR with U6 as control. (B) miR-216b expression levels in glioma cell lines (U87, T98G, U251 and LN229), and NHA cells were confirmed using RT-qPCR analyses. Experiments were performed at least 3 times for data analysis; *P<0.05, **P<0.01 and ***P<0.001.

Figure 2. Ectopic expression of miR-216b suppresses the proliferation, migration and invasion in vitro. (A) miR-216b expression was confirmed by RT-qPCR analysis after treatment with miR-216b mimics or NC in U87 and U251 glioma cells. (B) Glioma cell viability was detected using MTT assay. (C) Glioma cells infected with negative control (NC) or miR-216b were incubated for 2 week, and colony formation was conducted. (D and E) Glioma cell invasion abilities were evaluated by migratory and Transwell assays. (F) Western blot analysis of proliferation- and invasion-related proteins, Ki-67, MMP-2 and MMP-9 was assessed. Experiments were performed at least 3 times for data analysis; *P<0.05, **P<0.01 and ***P<0.001.
was significantly decreased when compared with this number in the NC group in the U87 and U251 cells. The experiment demonstrated that miR-216b also markedly (P<0.05) inhibited the migration ability of glioma cells (Fig. 2D and E). To validate that miR-216b influences cell proliferation and invasion, western blot analysis showed increased expression levels of Ki-67, MMP-2 and MMP-9, specific markers of cell proliferation and invasion, in the U87 and U251 cells following miR-216b deregulation (Fig. 2F). These results demonstrate that miR-216b inhibits cell proliferation and invasion of glioma cells.

FoxM1 is a direct target of miR-216b in glioma cells. To identify the mechanisms by which miR-216b suppresses glioma cell growth and invasion, we searched the Sanger microRNA database. Based on algorithm prediction, we observed that the ‘seed sequence’ of miR-216b matched the 3'UTR of FoxM1 mRNA (Fig. 3A). To test whether FoxM1 is regulated by miR-216b, we performed western blot analysis and RT-qPCR analyses in U87 and U251 cells, and found that endogenous FoxM1 expression was decreased in accordance with miR-216b upregulation (Fig. 3B and D). To determine whether FoxM1 is a direct target of miR-216b, we then constructed FoxM1-wild and FoxM1-mut luciferase reporter vectors. Then, we co-transfected the miR-216b overexpression plasmid with FoxM1-wild or FoxM1-mut constructs into the U87 and U251 cells. The assays showed that the luciferase activity of wild-type 3'UTR of FoxM1 significantly decreased in cells transfected with miR-216b, and was recovered by FoxM1-mut (Fig. 3C). Additionally, a significant negative correlation between miR-216b expression and FoxM1 protein expression was observed in glioma tissues (r=-0.3715, P=0.0257; Fig. 3E). Taken together, we conclude that miR-216b directly modulates FoxM1 expression via binding the 3'UTR of FoxM1.

Expression of FoxM1 overrides the effects of miR-216b in inhibiting glioma cell proliferation, migration and invasion. To determine whether FoxM1 is involved in the antitumour effects of miR-216b, the expression of FoxM1 was silenced by RNAi in the U87 cells (Fig. 4A and B). As shown in Fig. 4C-E, specific knockdown of FoxM1 by RNAi suppressed the ability of proliferation, migration and invasion in the U87 cells.

To define the importance of FoxM1 in miR-216b-mediated cell survival and invasion, we constructed a FoxM1-expression plasmid lacking the 3'UTR region. Rescue experiments showed that co-transfection of FoxM1 lacking 3'UTR significantly abolished miR-216b-induced inhibition of glioma cell
Figure 4. FoxM1 impacts proliferation and invasion of glioma cells. (A and B) After transfection with FoxM1-Si, the FoxM1 expression level was determined by RT-qPCR and western blot analysis. (C) The proliferation ability of glioma cells was regulated by FoxM1-Si. (D and E) FoxM1-Si inhibited the migration and invasion abilities of the glioma cells. Experiments were performed at least 3 times for data analysis; *P<0.05, **P<0.01 and ***P<0.001.

Figure 5. miR-216b overexpression blocks the invasive effects of FoxM1 on U87 cells. (A) The protein expression of FoxM1 in U87 cells was detected by western blot analysis. Cells were co-transfected with miR-216b mimics and FoxM1 plasmid vector or NC. (B-D) The growth, migration and invasion abilities were assessed by MTT, wound healing and Transwell assay, respectively. Experiments were performed at least 3 times for data analysis; *P<0.05, **P<0.01, ***P<0.001.
growth (Fig. 5B). U87 glioma cells were transfected with NC, FxoM1, miR-216b mimics or miR-216b plus FoxM1, and the expression of FoxM1 was detected by western blot assay (Fig. 5A). Meanwhile, miR-216b-mediated inhibition of cell migration and invasion was impeded by FoxM1 restoration in the U87 glioma cells transfected with miR-216b and FoxM1 (Fig. 5C and D). These results indicate that the effects of miR-216b on glioma cell biology were partially mediated by FoxM1.

**Discussion**

miRNAs have been identified to play a vital role in tumorigenesis since they can participate in many biological processes, such as cell proliferation, differentiation and invasion in a variety of cancer types. The loss of miR-216b expression has been reported in liver and breast cancer, pancreatic ductal adenocarcinoma, nasopharyngeal carcinoma and colorectal cancer (19-23). It was reported that miR-216b inhibited cell proliferation, metastasis and invasion by regulating PKC-α and KRAS in nasopharyngeal carcinoma (24), and HBx-miR-216b-IGF2bP2 signaling pathway in hepatocellular carcinoma (22). These results recognized miR-216b as a new tumour regulatory molecule.

However, the mechanism involved in the regulation of the growth of glioma by miR-216b remains unknown. In the present study, we first detected the downregulation of miR-216b in glioma tissues and cells and then confirmed the inhibitory effect on proliferation and invasion by miR-216b in glioma U87 and U251 cells by in vitro assays. These results provide us elementary evidence that miR-216b may exert a tumour-suppressor function in glioma.
FoxM1 is a protein involved in proliferation and cell cycle as a proto-oncogene (16), which is overexpressed in many human cancers, including glial tumours (25). FoxM1 belongs to the Forkhead Box superfamily of transcription factors, which regulates cell proliferation, differentiation, apoptosis and invasion (26). Upregulated FoxM1 expression is found in several types of cancers and plays a key role in G1/S and G2/M transition as a cell cycle-regulatory protein (27). Recently a study showed that FoxM1 regulates glucose metabolism in epithelial ovarian cells by targeting GLUT1 and HK2 transcription (28). In renal cell cancer cells, FoxM1 was found to regulate the cell cycle by activating PLK1 (29), and promoted colorectal cancer cell invasion and migration by regulating HSPA5 trans-activation (30).

MMP-2 plays a vital role in mesenchymal phenotypes in glioblastoma. Previous research has demonstrated that recurrent glioblastoma exhibits a mesenchymal subtype and the levels of MMP-2 are directly correlated with mesenchymal transition (31). TGF-β levels of MMP-2 are directly correlated with mesenchymal colorectal cancer cell invasion and migration by regulating epithelial ovarian cells by targeting GLUT1 and HK2 transcription (28). In renal cell cancer cells, FoxM1 was found to regulate TGF-β signaling by interacting with Smad3 in glioblastoma and MEF cells (34,35). Overexpression of FoxM1 promotes EMT and metastasis of HCC by targeting SNAI1, which plays a critical role in FoxM1-mediated EMT (33). The high FoxM1 expression and low E-cadherin expression in gastric cancer tissues play a critical role in the development and progression of gastric cancer (34). WNT/β-catenin and transforming growth factor (TGF-β)/SMAD signaling pathways which act downstream of FOXM1 targets, drive cancer progression by inducing EMT (35). FoxM1 overexpression is responsible for the acquisition of EMT and the CSC phenotype, which is in part regulated by miR-200b (36). These results indicate that miR-216b may play a vital role in tumour cell migration and the process of EMT.

Based on the results of bioinformatic analysis, we detected and verified that FoxM1 is a direct target gene of miR-216b in human glioma cells. In the present study, we found that overexpression of miR-216b reduced glioma cell proliferation and invasion. Thus, we conclude that miR-216b may have a role in the treatment of human glioma. Additionally forced expression of FoxM1 lacking the 3’UTR impeded the inhibition of proliferation and invasion by miR-216b overexpression, which indicates that FoxM1 is a potential target of miR-216b in human glioma. These data implicate that miR-216b has a potential role in the treatment of human glioma.

In conclusion, the present study demonstrated that miR-216b is downregulated in glioma and overexpression of miR-216b suppressed the proliferation, migration and invasion abilities by directly targeting FoxM1. These results reveal that re-expression of miR-216b may be a promising therapeutic target for glioma.

References


