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# Suppression of the biological activity of neuroglioma cells by down-regulation of miR-1

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**ABSTRACT.** Neuroglioma is associated with high rates of malignancy, metastasis, and recurrence. Recently, research on the roles of microRNAs (miR) in cancer prognosis has formed an important area of research as differential expression of miRNAs has been observed in different cancers. However, the detailed mechanism by which miRNAs regulate glioma remains unknown. Thus, we investigated the effect of miR-1 on human glioma by inhibiting the expression of miR-1. Anti-miR-1, an anti-sense oligonucleotide against miR-1, significantly reduced the level of miR-1 in the human glioma cell line U87 ($P < 0.05$). Further, cell proliferation and colony formation assays were used to determine the effect of miR-1 on cell growth. The scratch assay and cell migration assay were performed to evaluate cell invasion. Our data demonstrated that the growth of glioma cells was impeded due to the decrease in miR-1 levels, compared to the untransfected control cells ($P < 0.05$). Interestingly, the invasion and mobility of the cells were also retarded after transfection with anti-miR-1 ($P < 0.05$). In conclusion, our results indicate that the down-regulation of miR-1 significantly inhibited cell

proliferation and reduced cell motility, suggesting that anti-miR-1 could be used as a therapeutic intervention tool to counter the activity of neuroglioma cells.

**Key words:** microRNA-1; Neuroglioma; Cell proliferation; Cell migration; Cell motility

## INTRODUCTION

Neuroglioma is the most common sub-type of solid tumors and is ranked as the second-most lethal solid tumor worldwide (Sarli and Giannis 2008, Xu et al., 2011, Marra et al., 2013). Symptoms of neuroglioma may be subtle and worsen gradually, or may it may present as an acute illness. Headaches are common, along with several cases of movement disorder, auditory and speech malfunction, and confused thinking (Lang et al., 1993). The exact mechanism of occurrence of neuroglioma remains unclear. Heredity, diet composition, electromagnetic radiation, and microbial infection are proposed as risk factors in the progression of the disease (Reuss and von Deimling 2009). The therapy for neuroglioma varies according to the location, cell type, and grade of the malignancy. Besides surgery, current treatment mainly focuses on chemotherapy and radiotherapy. Temozolomide, for instance, was shown to traverse the blood-brain barrier, and therefore, is currently being used to treat high-grade tumors (Morr et al., 2016). However, various side effects and adverse prognosis exist, such as the unhindered proliferation of tumor stem cells that aggravate recurrence, as well as metastasis to other solid organs such as lymph nodes after enhancement of cell motility (Liu et al., 2013).

With the advancement of molecular microbiology in recent years, various novel methods have been developed for the treatment of glioma. RNA interference (RNAi), for example, has been widely used to treat glioma on the genetic level (Judge et al., 2009; Yin et al., 2010; Deezagi et al., 2012). RNAi involves hybridization of the coding sequences with the non-sense small RNA fragments that causes targeted silencing of mRNA, and thereby, gene expression. RNAi has become an important subject in both basic research and clinical treatment (Peroukides et al., 2010; Sun et al., 2012), and RNAi-based treatment for glioma is currently a new research objective. As a muscle-specific miR, miR-1 is associated with heart development and arrhythmia/myocardial hypertrophy and is a potential therapeutic tool in medical research (Oh et al., 1999; Tai et al., 2012). A recent study demonstrated that high expression of *miR-1* was observed in esophageal squamous cell carcinoma (ESCC) tissues and cell lines. Cell proliferation, invasion, metastasis, and progression of ESCC were affected by targeting of *LASP1* and *TAGLN2* by miR-1 (Du et al., 2016). In contrast, in colorectal carcinoma (CRC) tissues and cell lines, the expression of *miR-1* was reduced, and miR-1 contributed to the restriction of CRC metastasis via the MAPK and PI3K/AKT pathway (Xu et al., 2014). These findings indicate that miR-1 plays a critical role in cancer progression and is a potential candidate for the molecular therapy of cancer. However, the exact function of miR-1 in the occurrence and progression of neuroglioma is unknown. Additionally, microRNAs such as *miR-181a* can be down-regulated to activate caspase-3 expression in the treatment of neurological disorders (Ren et al., 2016). miR-133 can inhibit cell migration and invasion of the pituitary adenoma by directly targeting *FOXC1*, implying that microRNAs are potential therapeutic targets for treatment of neurological diseases (Wang et al., 2016). In this study, we sought to investigate the role of miR-1 in the human neuroglioma U87 cell line.

## MATERIAL AND METHODS

### Reagents

Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Opti Minimum Essential Medium (MEM) and Roswell Park Memorial Institute (RPMI) 1640 media were purchased from Gibco (Grand Island, NY, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). 0.5 g MTT was dissolved in 100 mL PBS, filtered through a 0.22-mm membrane, and stored in the dark at 4°C. The anti-miR-1 (miR-1 antisense oligonucleotide) and a nonspecific anti-miR control were purchased from GenePharma (Shanghai, China). miR-1 primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

### Stable transfection of U87 cells with anti-miR-1

U87 cells at log-phase of growth were seeded in 6-well plates (with 3 x $10^5$ to 5 x $10^5$ cells per well) without antibiotics for 24 h prior to transfection, resulting in 60-80% confluency. miRNAs were transfected at a working concentration of 100 nM using Lipofectamine 2000 reagent according to the manufacturer's instructions. Anti-miR-1 (miR-1 inhibitor) was mixed with serum-free Opti MEM medium containing Lipofectamine 2000 according to the manufacturer's recommendation. After 6 h at 37°C, the medium was changed, and the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS. After 48 h, RNA was extracted for detecting the levels of miR-1. Cells transfected with the non-specific anti-miR-1 oligonucleotide served as a negative control (NC), whereas, untransfected cells were used as blank control.

### RNA extraction

Total RNA was extracted and purified from cultured U87 cells by TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan). The concentration and purity of RNA were measured by Nano Drop 2000 (Thermo Fisher Scientific, USA); a value of 1.9-2.0 of the ratio of the absorbance at 260 nm and 280 nm (A260/280) indicated highly pure RNA.

### Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed in a 25-μL reaction volume containing 2 mg RNA, 1 μL oligo dT (50 μM), 2 μL dNTP (10 mM) and DEPC-treated water by using SuperScript® III One-Step RT-PCR System with Platinum®Taq (Thermo Fisher Scientific, Waltham, MA, USA). *In vitro* reverse transcription was initiated by denaturation at 65°C for 5 min, followed by addition of 4 μL synthesis buffer (containing 5 x cDNA), 1 μL 0.1 M DTT, 40 units RNase Out, 1 μL DEPC-treated water, reverse transcriptase (0.5 μL) (ThermoScript, USA), and 15 units reaction buffer. The reverse transcription reaction was incubated at 50°C for 1 h, followed by incubation at 85°C for 5 min. RNaseH was added to inactivate the residual RNA for 20 min at 37°C.

### Quantitative PCR (qPCR)

The Master Mix (Superarray, USA) was used for qPCR in a 25 μL volume containing 12.5 μL SYBR Green Master Mix, 10.5 μL double distilled water, 1 μL template cDNA, and 1

μL PCR primer (10 μM). PCR conditions were: pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec. The melting curve analysis was used to determine the relative expression levels. The primer sequences were: *miR-1*-F: 5'-GAGGAGGCTGGAGAA-3'; *miR-1*-R: 5'-GGTGGAATGTAAAGAAGTC-3'; *GAPDH*-F: 5'-AGAAGGCTGGGGCTCATTTG-3'; *GAPDH*-R: 5'-CTCGCTCCTGGAAGATGGTG-3'. GAPDH was used as an internal control.

## 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The U87 cells were prepared as single cell suspensions in culture medium containing 10% FBS, and were seeded on 96-well plates, with 0.2 mL culture in each well (containing 5000-6000 cells). After the attachment and growth of the cells, the MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well (20 μL). After 4 h of incubation, supernatants were discarded. Cells were recentrifuged and were mixed with 150 μL dimethyl sulfoxide. The crystal was dissolved by vortexing for 10 min. Absorbance at 490 nm was measured to plot the growth of cells as a function of time.

## Clonal formation assay

Cell suspensions were seeded in culture dishes (about 100 cells) until they formed visible colonies. Supernatants were discarded by rinsing twice with PBS (pH 7.4). 4% paraformaldehyde was added to fix the cells for 15 min. The fixing buffer was then removed and crystal violet was added and incubated for 10-30 min. The excess dye was washed away under tap water and the plates were air-dried. The clonal formation assay reflected both the colony-dependency and proliferation features of the cells.

## Scratch assay

The scratch assay was used to observe the motility of the tumor cells with attachment growth. Tubular epithelial cells (TEC) at log-phase was seeded in 6-well plates, with 3 x $10^5$ to 5 x $10^5$ cells per well. A sterilized tooth-stick was used to draw a "+" shape on the culture plate. Images were taken every 12 h for observing cell growth.

## Cell migration assay

Neuroglioma cells were seeded in 6-well plates (1 mL each). Four plates were used for each group. When the cells were 70% confluent, Lipofectamine 2000 was used to transfect 100 nmol anti-miR-1 or anti-miR NC. After 72 h, the cells were digested by trypsin and were added into the upper chamber of the Trans-well, whose lower chamber was filled with Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS. After 24 h of incubation, the trans-well chamber was washed, fixed, and stained by crystal violet. An inverted microscope (Olympus, Japan) was used to count the number of perforated cells in five different fields for calculating the average cell number in each field.

## Statistical analysis

The SPSS 11.0 software package was used to process the collected data. The student's

*t*-test was used to compare the means. Pearson analysis and the chi-square test were used to analyze correlations. A statistical significance was defined when $P < 0.05$.

## RESULTS

### Down-regulation of miR-1 in glioma cells

To construct a glioma cell line with down-regulated miR-1, U87 cells were transfected with anti-miR-1. qPCR (Figure 1) showed that the expression of miR-1 in the cells transfected with anti-miR-1 was significantly lowered (4.3 ± 1.5) than that in the NC group (25.2 ± 1.9) or blank control (25.9 ± 1.3) ($P < 0.05$), while no remarkable difference was observed between the NC and the blank control ($P > 0.05$), suggesting that the reduced level of miR-1 in U87 cells was anti-miR-1 specific.

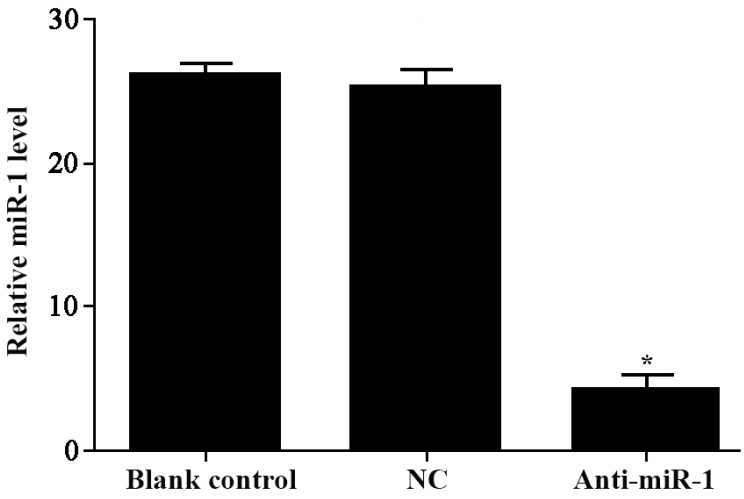


**Figure 1.** Expression of miR-1 analyzed by RT-PCR was decreased in glioma cells. Bar graph depicting the levels of miR-1 normalized by that of GAPDH. Data are reported as means ± SD. *$P < 0.05$ compared with the blank control.

### Down-regulation of miR-1 inhibited the growth of glioma cells

The MTT assay was used to detect the effect of miR-1 on cell growth at 24, 48, and 72 h after the transfection with anti-miR-1 or anti-miR NC. Results showed that the proliferation of the cells in the anti-miR-1 group was significantly inhibited (0.31 ± 0.11 at 24 h; 0.49 ± 0.13 at 48 h; 0.91 ± 0.15 at 72 h) compared to that of the cells without any treatment ($P < 0.05$), whereas the proliferative abilities in the NC group (1.08 ± 0.16 at 24 h; 1.63 ± 0.14 at 48 h; 2.69 ± 0.18 at 72 h) or blank control (1.05 ± 0.13 at 24 h; 1.51 ± 0.15 at 48 h; 2.43 ± 0.20 at 72 h) were approximately similar (Figure 2A). This was further validated by the clonal formation assay, which demonstrated that the down-regulation of miR-1 suppressed cell growth compared to that in the blank or the negative control, indicating that miR-1 plays a positive role in the proliferation of the glioma cells (Figure 2B).

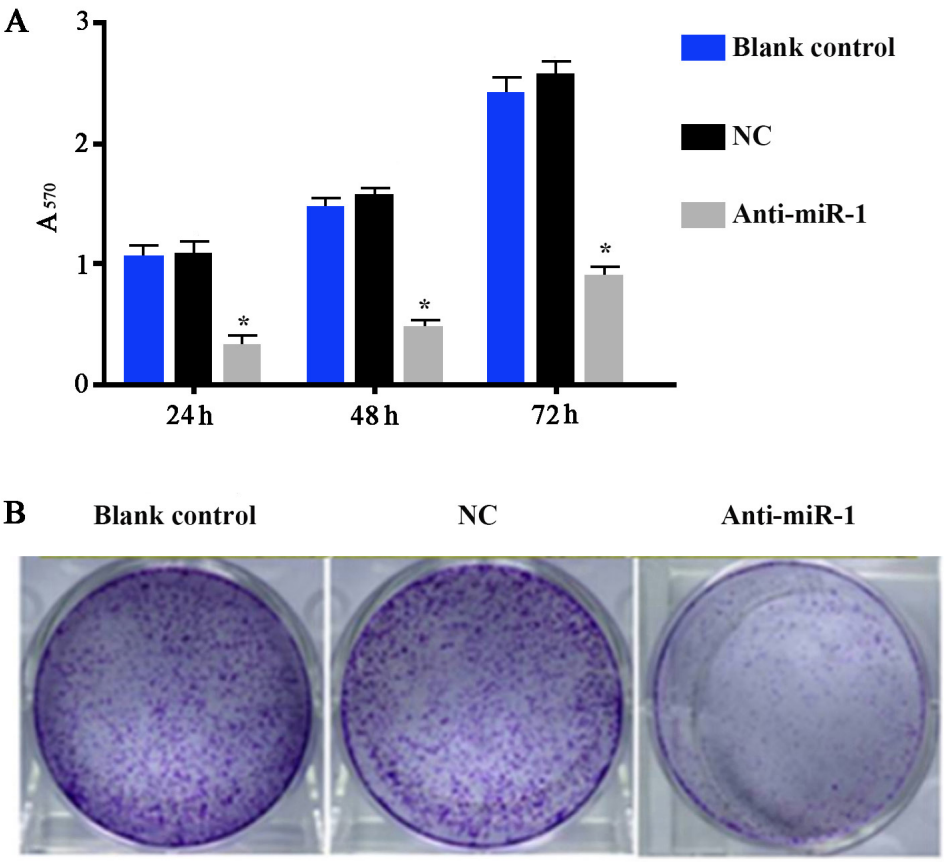


**Figure 2.** Cell proliferation was inhibited by down-regulation of miR-1. **A.** Absorbance values at 490 nm were determined to evaluate cell growth after the MTT assay. *P < 0.05 compared with blank control. Each bar represents means ± SD; **B.** Images showing the results of the clonal formation assay. Blue dots indicated the cell colonies. All least three independent experiments were performed.

## Reducing the level of miR-1 impeded cell motility and migration

We utilized the scratch assay to detect cell mobility and invasion at 0, 12, and 24 h post-transfection. Our data revealed an increase in the number of migrated cells in the NC group. However, the number of migrated cells was lower after treatment with anti-miR-1 (Figure 3A). At the same time, a parallel assay for detecting cell motility demonstrated that the migration was relatively retarded after the transfection with anti-miR-1 72 h after the transfection, compared to that observed in the blank or negative control (Figure 3B). The data also showed that there was a statistical decrease in the number of migrated cells in anti-miR-1 group (11.2 ± 1.8), compared to that in the blank control (22.5 ± 2.6) (P < 0.05) (Figure 3C). Together, these results suggested that the invasiveness of the glioma cells was alleviated by the reduction in the level of miR-1.

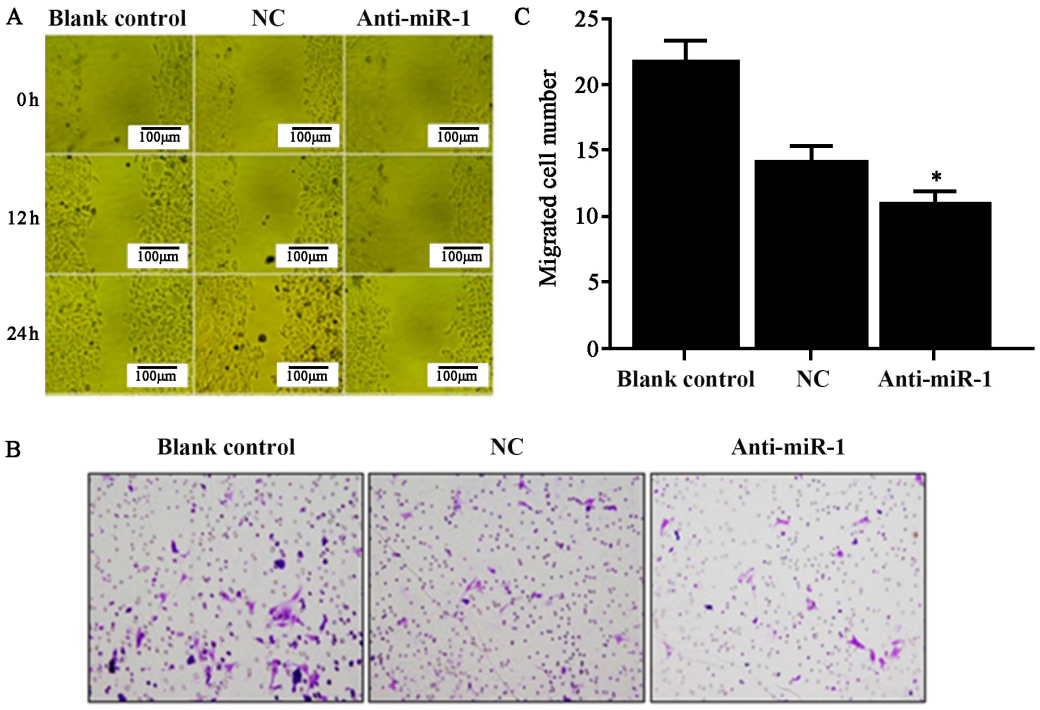


**Figure 3.** Effects of reduced miR-1 level on the migration and invasion of glioma cells. **A.** Invasion of cells was determined by the scratch assay; scratch healing was observed at 0, 12, 24 h post- transfection; **B.** Cell migration was observed by the trans-well assay after 72 h of transfection; **C.** Histogram showing the average number of perforated cells in five fields. *P < 0.05 compared with the blank control. Data are represented as means ± SD. All least three independent experiments were performed.

## DISCUSSION

The pathogenesis of neuroglioma, a highly malignant tumor, is complicated by the involvement of aberrant gene expression which affects cell growth, invasiveness, and motility. The hyperactive tumor cell motility is one of the main reasons for the aggravation of tumor growth. Recently, miRNAs have been shown to be involved in the proliferation, invasion, and apoptosis of various tumors, and the enhancement or suppression of tumor motility was influenced by the modulation of small RNA levels (Krol et al., 2010). For instance, miR-129 affected the proliferation of pulmonary carcinoma via the down-regulation of Cdk6 in a mouse lung cancer cell line (Cheng et al., 2005). Studies have demonstrated that tumor cell growth and motility, as well as infiltration of neuroglioma, are closely related to tumor metastasis (Chen et al., 2006; Atkinson et al., 2010). However, the exact roles of microRNAs in the aggravation of tumor cell mobilization are still unknown.

Previous studies have found that microRNAs could inhibit intestinal tumor cell growth and elevate levels of apoptosis, suggesting that the effective inhibition of tumor cell growth by targeted microRNAs can be considered for treatment of tumors (Ikeda et al., 2007; Mangoni and Nargeot, 2008). Several findings reported that miRNAs accelerated the invasiveness of neuroglioma, while others demonstrated an opposite role of miRNAs in the

inhibition of cancer migration and invasiveness (Yang et al., 2007). For example, miR-146b can inhibit migration and invasion of the neuroglioma cells (Buck et al., 1999), and miR-133 suppressed cell migration and invasion of the pituitary adenoma (Wang et al., 2016). In contrast, miR-125b enhanced skin tumor and prolonged malignant cell survival (Zhang et al., 2014). Moreover, miR-29a regulated the heat shock protein 47 to enhance glioma tumor growth and invasion (Zhao et al., 2014).

Neuroglioma is the leading cause of intracranial tumor. As a small molecule RNA present in muscles, miR-1 has been sparsely studied in neuroglioma cells. As previous studies indicated that the expression of miR-1 was higher in neuroglioma cells compared to that in normal cells (Cao et al., 2012), we investigated whether the suppression of miR-1 could affect the proliferation of the glioma cells. We down-regulated the level of miR-1 in the human neuroglioma cell U87 by transfection with anti-miR-1. Notably, reducing the level of miR-1 impeded cell proliferation and retarded colony formation. Additionally, the lowering of miR-1 expression inhibited the invasiveness and motility of the tumor cells. Overexpression of miRNAs, such as miR-200c, miR-141, miR-126, and miR-132, suppressed glioma cell growth and migration (Guo et al., 2016; Li et al., 2016; Wang et al., 2015). Interestingly, our result demonstrated that reducing the expression of miR-1 had a similar inhibitory role in the glioma cells, which is consistent with a previous finding that shows that the alleviation of *miR-1* expression inhibited colorectal carcinoma metastasis. This indicates that the progression of specific cancers may be restricted by anti-miR-1 (Xu et al., 2014). However, *in vivo* experiments are required to confirm the tumor-suppressive effects of anti-miR-1, identify its potential downstream targets, and understand its molecular mechanisms of action.

Taken together, our preliminary data highlighted the role of miR-1 in the metastasis of glioma cells and provided a potential therapeutic target for the treatment of high-grade glioma.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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