miR-133b inhibits glioma cell proliferation and invasion by targeting Sirt1

Chuntao Li[1], Zhixiong Liu[1], Kui Yang[1], Xin Chen[1], Yu Zeng[1], Jinfang Liu[1], Zhenyan Li[1], Yunsheng Liu[1]

[1]Department of Neurosurgery, Xiangya Hospital of Central South University, Changsha, 410008 Hunan, China

Correspondence to: Zhixiong Liu, **email:** zxliu_csu@sina.com

ABSTRACT

MicroRNAs (miRs) are a class of small non-coding RNAs that function as mediators of gene expression. Dysregulations of miRs have been implicated in the development and progression of glioma. In the present study, we investigated the role of miR-133b in mediating the proliferation and invasion of glioma cells, and the potential mechanism. Real-time RT-PCR results showed that miR-133b expression was significantly decreased in glioma tissues compared with normal brain tissues. Luciferase reporter assay further identified silent information regulator 1 (Sirt1) as a novel direct target of miR-133b in glioma U87 cells. Overexpression of miR-133b suppressed Sirt1 expression and reduced the proliferation and invasion of U87 cells, which could be partly rescued by forced expression of Sirt1. In addition, the Sirt1 mRNA level was significantly higher in glioma tissues than in normal brain tissues, and was inversely correlated with miR-133b level in glioma tissues. In summary, our study sheds light on the regulatory mechanism of miR-133b in glioma growth and metastasis *via* direct mediation of Sirt1 expression, and suggests that Sirt1 may serve as a potential therapeutic target for glioma.

INTRODUCTION

Glioma is the most common malignant brain tumor, accounting for about 80% of brain tumors; advanced glioma has a high mortality rate [1–3]. Although great progresses on the surgical resection, chemotherapy and radiotherapy have been made, the prognosis of glioma remains poor [4]. Therefore, effective diagnostic and therapeutic strategies for glioma are urgently needed.

MicroRNAs (miRs) are a type of short non-coding RNAs, and act as key regulators of gene expression by directly binding to the 3′-untranslational region (UTR) of their target mRNAs [5]. miRs can lead to translation repression or mRNA degradation, and thus generally suppress the protein expression of their targets [5, 6]. Through mediation of their targets' expression, miRs have been shown to be implicated in several biological processes, including cell proliferation, cell cycle progression, differentiation, apoptosis and motility [7–10]. Moreover, miRs play important regulatory roles in the development and

progression of human cancers, as miRs target many tumor suppressors and oncogenes [11–14]. Recently, miR-133b dysregulation has been shown to play a key role in several human cancers [15–17]. miR-133b expression, which was associated with overall survival and metastasis, was greatly downregulated in colorectal cancer [18]. In addition, miR-133b expression inhibited the proliferation of colorectal cancer cells *in vitro* and *in vivo* by directly targeting the receptor tyrosine kinase MET [19]. miR-133b could act as a tumor suppressor in esophageal squamous cell carcinoma by inhibiting FSCN1 expression [20]. miR-133b was also reported to be implicated in glioma [21]. Wang et al. showed that miR-133b was markedly downregulated in clinical glioblastoma specimens, and contributed to arsenic-induced apoptosis in U251 glioma cells by targeting the hERG channel [21]. However, the exact role of miR-133b in mediating the proliferation and invasion of glioma cells and the underlying mechanism remain largely unknown.

In the present study, we aimed to reveal the exact role of miR-133b in glioma. Our data showed that miR-133b was downregulated in glioma and suppressed the

proliferation and invasion of glioma U87 cells, at least partly by targeting silent information regulator 1 (Sirt1).

RESULTS

MiR-133b is significantly downregulated in glioma compared with normal brain tissues

To study the role of miR-133b in glioma, we first examined the miR-133b levels in 21 glioma tissue specimens and 8 normal brain tissue specimens. Real-time RT-PCR data showed that miR-133b expression was significantly reduced in glioma tissues compared with non-tumor brain tissues ($P < 0.01$; Figure 1), suggesting that miR-133b downregulation may be involved in glioma development.

Sirt1 is a direct target of miR-133b in glioma U87 cells

The putative targets of miR-133b were further analyzed using bioinformatical prediction and Targetscan (http://www.targetscan.org/). Our data showed that Sirt1 was a putative target gene of miR-133b (Figure 2A). To confirm their relationship, wild-type or mutant Sirt1 3′-UTR (Figure 2B) was constructed and inserted into the psiCHECK™2 vector (Figure 2C). Then, a luciferase reporter assay was performed in glioma U87 cells. Our data showed that luciferase activity was only significantly downregulated in glioma U87 cells co-transfected with miR-133b mimic and wild-type Sirt1 3′UTR ($P < 0.01$). However, luciferase activity was unchanged in other groups (Figure 2D). Therefore, Sirt1 is a target gene of miR-133b in glioma cells.

As miRs negatively mediate the protein expression of their target genes, we examined the effects of miR-133b on the protein expression of Sirt1 in glioma cells. U87 cells were transfected with miR-133b mimic or inhibitor. After transfection, real-time RT-PCR was conducted to examine the miR-133b level. Transfection with miR-133b mimic led to a significant upregulation of miR-133b, while transfection with miR-133b inhibitor resulted in a significant decrease in miR-133b ($P < 0.01$; Figure 2E). The protein level of Sirt1 was further examined by western blot. As shown in Figure 2F and 2G, the protein level of Sirt1 was significantly reduced in miR-133b-overexpressing U87 cells, but increased after miR-133b knockdown, compared with the control group ($P < 0.01$). Therefore, miR-133b negatively regulates the protein expression of Sirt1 in U87 cells by directly binding to the 3′-UTR of its mRNA.

MiR-133b suppresses glioma cell proliferation and invasion

We further investigated the effects of miR-133b on glioma cell proliferation and invasion. MTT assay data showed that miR-133b overexpression significantly inhibited U87 cell proliferation, while miR-133b knockdown enhanced U87 cell proliferation ($P < 0.01$; Figure 3A), suggesting that miR-133b may suppress glioma growth. Transwell assay data further indicated that miR-133b overexpression significantly inhibited U87 cell invasion, while miR-133b knockdown enhanced U87 cell invasion ($P < 0.01$; Figure 3B). These findings suggest that miR-133b may play an inhibitory role in glioma metastasis.
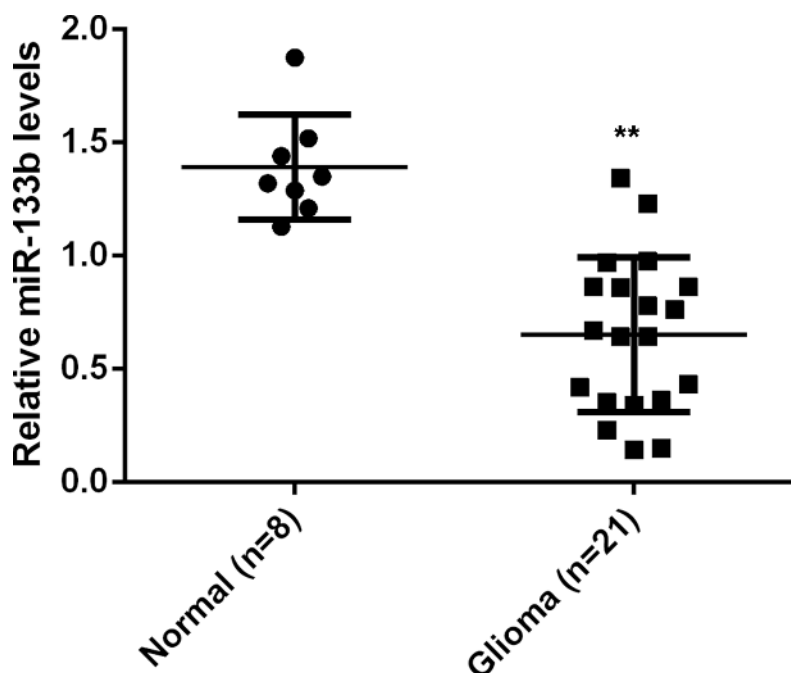

Figure 1: Real-time RT-PCR was conducted to examine the relative miR-133b levels in 21 glioma specimens and 8 normal brain tissue specimens. **$P < 0.01$ vs. Normal.

Sirt1 is involved in miR-133b-mediated glioma cell proliferation and invasion

Based on the above data, Sirt1 is involved in miR-133b-mediated glioma cell growth and metastasis. To rescue the suppressive effect of miR-133b upregulation on Sirt1 expression, miR-133b-overexpressing U87 cells were transfected with Sirt1-overexpressing plasmid. Then, western blot was conducted to examine the Sirt1 protein. Our data showed that the Sirt1 protein level was higher in the miR-133b + Sirt1 group than in the miR-133b group (Figure 4A). MTT assay data showed that cell proliferation
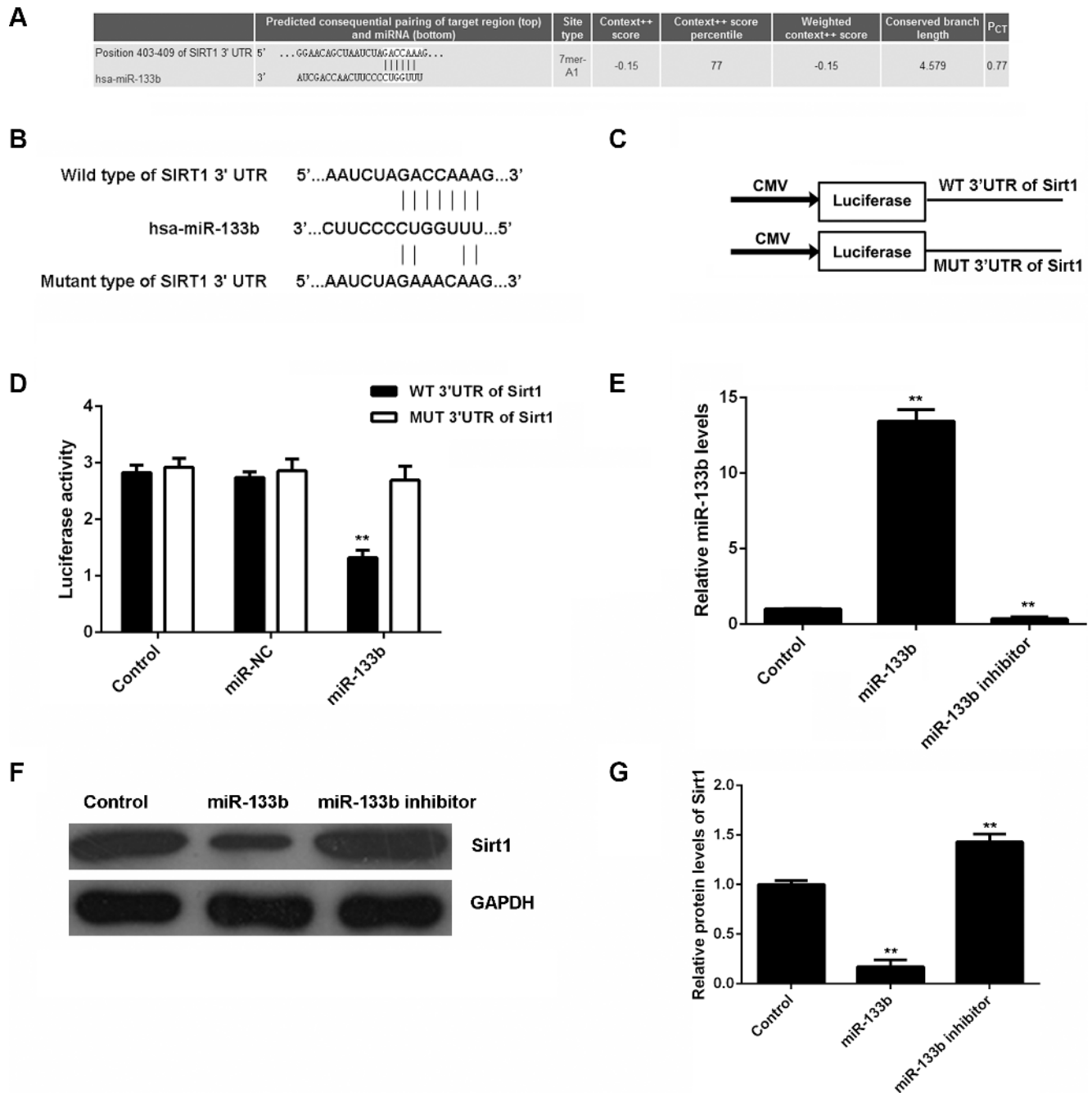

Figure 2: miR-133b specifically targets Sirt1 gene. (**A**) Targetscan software predicts that Sirt1 is a direct target gene of miR-133b. (**B**) The wild-type (WT) or mutant (MUT) binding sequences of miR-133b on Sirt1 3'-UTR are shown. (**C**) The WT or MUT Sirt1 3'-UTR was constructed and inserted into the psiCHECK ™2 luciferase reporter vector. (**D**) The luciferase activity was significantly downregulated in glioma U87 cells co-transfected with miR-133b mimic and WT Sirt1 3′UTR vector, but was unchanged in other groups. Control: U87 cells transfected with WT or MUT Sirt1 3′UTR vector. **$P < 0.01$ vs. Control. (**E**) Real-time RT-PCR was used to examine the miR-133b levels in U87 cells transfected with miR-133b mimic or inhibitor, and (**F, G**) western blot was performed to examine the Sirt1 protein levels in each group. Control: non-transfected U87 cells. **$P < 0.01$ vs. Control.

was enhanced in the miR-133b+Sirt1 group (Figure 4B). Transwell assay showed that cell invasion was promoted in the miR-133b+Sirt1 group (Figure 4C). Therefore, forced expression of Sirt1 could partly rescue the suppressive effects of miR-133b on the proliferation and invasion of U87 cells.

To examine the relationship between miR-133b and its target gene Sirt1, we performed real-time RT-PCR to examine the mRNA level of Sirt1 in glioma and normal brain tissues. The Sirt1mRNA was significantly higher in glioma than in normal brain tissues (Figure 5A). Moreover, Sirt1mRNA was inversely correlated with miR-133b level in glioma tissues (Figure 5B). These data further supported that Sirt1 upregulation may contribute to miR-133b downregulation in glioma, which might further drive glioma growth and metastasis.
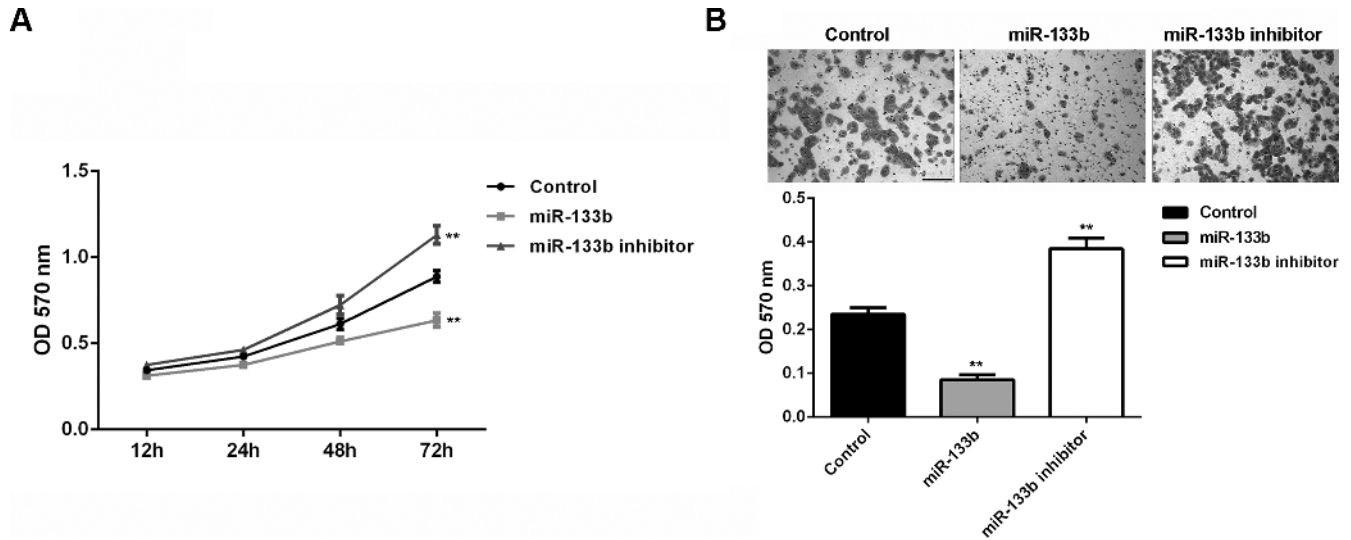

Figure 3: miR-133b overexpression could decrease the cell proliferation and invasion of glioma cells. (**A**) MTT assay and (**B**) transwell assay were conducted to determine the proliferation and invasion of U87 cells transfected with miR-133b mimic or inhibitor, respectively. Control: non-transfected U87 cells. **$P < 0.01$ vs. Control. bar = 100 μm.
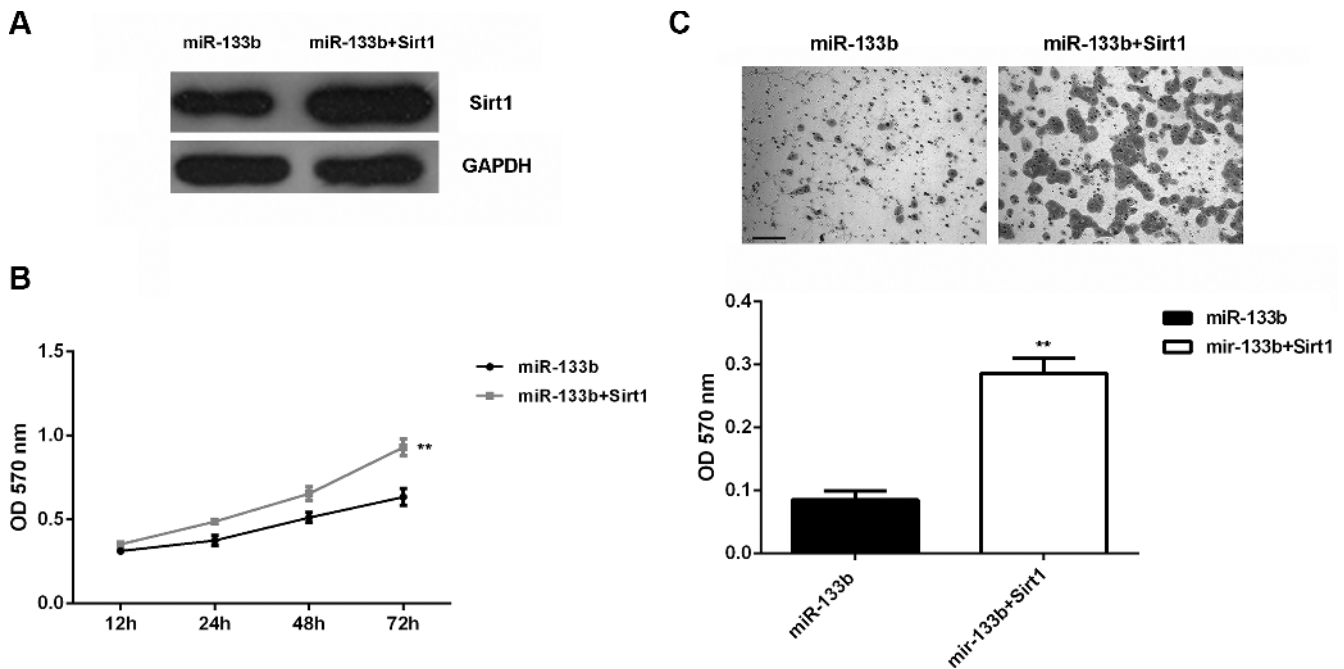

Figure 4: Forced Sirt1 expression partly abolished the effect of miR-133b on cell proliferation and invasion. (**A**) Western blot was performed to examine the Sirt1 protein levels in U87 cells transfected with miR-133b mimic, or co-transfected with miR-133b mimic and Sirt1-overexpressing plasmid (miR-133b + Sirt1). (**B**) MTT assay and (**C**) transwell assay were conducted to determine the cell proliferation and invasion in each group. **$P < 0.01$ vs. miR-133b. bar = 100 μm.

DISCUSSION

The precise role of miR-133b in the regulation of the malignant phenotypes of glioma cells remains largely unknown. In the present study, we found that miR-133b was significantly downregulated in 21 glioma specimens compared with 8 normal brain tissue specimens. Sirt1, an oncogene in glioma, was further identified as a direct target of miR-133b in U87 cells. The *in vitro* study showed that miR-133b could inhibit the proliferation and invasion of U87 cells, at least partly by directly inhibiting Sirt1 protein expression. We also found that Sirt1 mRNA was significantly increased and was inversely correlated with miR-133b level in glioma tissues.

Recently, miR-133b levels were found to be markedly decreased in glioblastoma, that is, high grade glioma [21]. In the present study, real-time RT-PCR data indicated that miR-133b was downregulated in glioma tissues compared with normal brain tissues. These data suggest that miR-133b may act as a tumor suppressor in glioma. We further used the glioblastoma U87 cell line to study the exact role of miR-133b in the regulation of cell proliferation and invasion. We transfected U87 cells with miR-133b mimic to upregulate miR-133b, and observed that miR-133b overexpression led to significant inhibition of cell proliferation and invasion. The inhibitory effects of miR-133b on cell proliferation and invasion were also reported in other cancers. Qiu et al. showed that miR-133b inhibited proliferation, migration, invasion and cell cycle progression by targeting transcription factor Sp1 in gastric cancer [15]. Zhao and colleagues reported that miR-133b was downregulated in osteosarcoma tissues, inhibited proliferation, migration and invasion, and promoted apoptosis of osteosarcoma cells [22]. Moreover, Wu et al. showed that miR-133b was downregulated in renal cell carcinoma, and suppressed the cell proliferation, migration and invasion by targeting MMP9 [23].

Sirt1 is a member of the mammalian sirtuin protein family, which functions as a conserved nicotinamide adenine dinucleotide (NAD)+-dependent deacetylase that is implicated in the modulation of transcriptional silencing and cell survival [24]. Sirt1 is involved in various biological processes, including cell growth, differentiation, apoptosis, autophagy, metabolism and aging and related diseases [25–31]. A dual role of Sirt1 has been reported in human cancers. Kim et al. suggested that Sirt1 acted as a tumor suppressor in luminal A breast cancer, and decreased Sirt1 expression was significantly associated with metastasis and poor survival [32]. In contrast, Sirt1 was upregulated in colorectal cancer, and Sirt1 level was correlated with the TNM stage and mutant P53 loss [33]. Lu et al. reported that Sirt1 repressed gastric cancer growth by counteracting STAT3 and NF-κB activation [34]. Thus, the role of Sirt1 is tumor-specific. The oncogenic role of Sirt1 in glioma has been a hot topic in recent years. Sirt1 was found to promote proliferation and inhibit apoptosis of glioma cells [35]. Knockdown of Sirt1 was proved to enhance the effectiveness of radiotherapy in CD133+ glioma cells *in vitro* and *in vivo* [36]. SIRT1 is also required for p53-dependent oncogenic transformation of neural stem cells [37]. In the present study, we found that Sirt1 overexpression reversed the miR-133b-mediated suppression of U87 cell proliferation and invasion, indicating that Sirt1 acts as a downstream effector of miR-133b in glioma cells.

Therefore, our findings revealed a tumor-suppressive role of miR-133b in glioma partly by targeting Sirt1, indicating that the miR-133b/Sirt1 axis may serve as a potential therapeutic target for treating such deadly disease. Furthermore, the diagnostic and prognostic potential of miR-133b in the carcinogenesis of glioma will be further examined in large-scale studies in future.
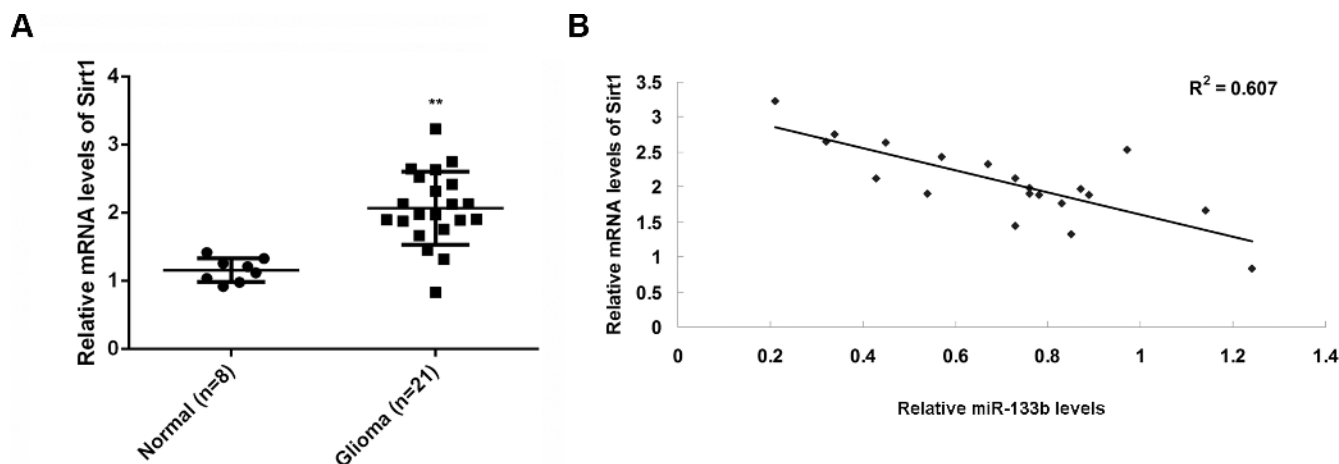

Figure 5: Sirt1 mRNA was negatively correlated with miR-133b level in tumor tissue. (**A**) Real-time RT-PCR was conducted to examine the relative mRNA levels of Sirt1 in 21 glioma specimens and 8 normal brain tissue specimens. **P < 0.01 vs. Normal. (**B**) The reverse correlation between the miR-133b levels and the Sirt1 mRNA levels in 21 glioma specimens.

MATERIALS AND METHODS

Tissue specimen collection

Glioma tissues (n = 21) and normal brain tissues (n = 8) were collected at Xiangya Hospital from April 2013 to February 2014. Tissues were immediately snap-frozen in liquid nitrogen after surgical removal and stored at −80°C before use. The histomorphology was pathologically confirmed. Our study was approved by the Ethics Committee of Xiangya Hospital of Central South University, Changsha, China. All written informed consents were obtained before the study began.

Cell lines and cell culture

The human glioma U87 cell line was purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) with 10% fetal bovine serum (FBS, Life Technologies), 100 IU/ml penicillin, and 100 IU/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Real-time RT-PCR assay

Total RNA of tissues or cells was extracted using Trizol Reagent (Life Technologies). A Reverse Transcription Kit (Life Technologies) was used to synthesize cDNA. For mRNA and miR detection, real-time PCR was conducted using a Q-PCR Detection Kit (Life Technologies) and miRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA)on an ABI 7500 thermocycler (Life Technologies), respectively. GAPDH or U6 gene was used as internal reference. The PCR reaction conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [38].

Transfection

Sirt1-overexpressing plasmid (Amspring, Changsha, China), scramble miR (Amspring), miR-133b mimic (Amspring), miR-133b inhibitor (Amspring) or Lipofectamine 2000 (Life Technologies) was diluted with serum-free DMEM. The transfection mixture was added into the cell suspension and incubated for 6 h. The transfection mixture was replaced with DMEM with 10% FBS. Cells were then cultured for 48 h before assays were performed.

Dual luciferase reporter assays

Wild-type (WT) Sirt1 3′-UTR was constructed using PCR and inserted into the psiCHECK ™2 vector (Promega, Madison, WI, USA). The construction of Mutant Sirt1 3′-UTR (MUT) was completed by a Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA),

and inserted into the psiCHECK ™2 vector. U87 cells were co-transfected with WT Sirt1-3′UTR or MUT Sirt1-3′UTR plasmid (100 ng) and scramble miR or miR-133b mimic (50 nM) using Lipofectamine 2000 for 48 h. A dual-luciferase reporter assay system (Promega) was used to determine renilla luciferase and firefly luciferase activity. Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot

The protein concentration was determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). Protein was separated with 10% SDS-PAGE, transferred to a PVDF membrane (Life Technologies), and then blocked in 5% nonfat dried milk. The PVDF membrane was incubated with rabbit anti-Sirt1 monoclonal antibody (1:100, Abcam, Cambridge, MA, USA) or mouse anti-GAPDH monoclonal antibody (1:100, Abcam), as an internal reference. After that, the PVDF membrane was incubated with mouse anti-rabbit secondary antibody (1:20000, Abcam). The immune complexes were detected using the ECL Western Blotting Kit (Pierce Chemical, Rockford, IL, USA). Image-Pro plus software 6.0 was used to analyze the relative protein expression, represented as the density ratio versus GAPDH.

Cell proliferation detection

MTT assay was used to examine cell proliferation. Different groups of U87 cells (10000) were plated into a 96-well plate, and were cultured at 37°C with 5% CO_2 for 12, 24, 48, or 72 h. Then, 20 μL of MTT (5 mg/mL, Life Technologies) was added. The formazan production was detected by determining the optical density (OD) at 570 nm using a Multiskan FC enzyme immunoassay analyzer (Thermo Fisher Scientific).

Cell invasion assay

The cell invasion assay was performed using transwell chambers (BD, USA) pre-coated with Matrigel. 300 μl of 5×10^5 cells/ml cell suspension was added into upper chamber, and 500 μl 10% FBS DMEM was in lower chamber. The cells that did not migrate or invade through the pores were wiped out. The filters were fixed in 90% alcohol and stained with 0.1% crystal violet, and were observed under an inverted microscope (Olympus, Japan). 0.5 g/L MTT was added and the optical density at 570 nm was measured using the Bio-Tek™ ELX-800™ Absorbance Microplate reader (Bio-Tek, USA). The relative cell invasive capacity was determined.

Statistical analysis

Continuous data are expressed as mean ± *SD*. Data analysis was performed using GraphPad Prism 6.0 software.

Student's *t*-tests or one-way ANOVAs were used to analyze the differences among groups. Statistical significance was indicated by a *P* value of < 0.05.

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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