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miR-132 weakens proliferation and invasion of glioma cells via the inhibition of Gli1

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Abstract. – OBJECTIVE: Transcriptional factor Gli1 in Hedgehog signal pathway facilitates epithelial mesenchymal transition (EMT) and is associated with invasion or proliferation of multiple tumor cells. The previous study showed the correlation between miR-132 down-regulation and glioma pathogenesis. We investigated the role of miR-132 in mediating Gli1 expression and in affecting proliferation or invasion of glioma cells.

PATIENTS AND METHODS: Dual luciferase reporter gene assay was used to confirm the targeted regulation between miR-132 and Gli1. Tumor tissues at different pathological grades (grade II, III and IV) were collected from glioma patients, in parallel with brain tissues from contusion surgery. The expression of miR-132 and Gli1 was measured by RT-PCR. Glioma cell line U251 was treated with miR-132 or si-Gli1 followed by measuring the expression of Gli1, E-cadherin, Vimentin and Cyclin D1. In addition, flow cytometry and transwell assay were performed to evaluate cell invasion potency.

RESULTS: Bioinformatics analysis showed the complementary binding sites between miR-132 and 3'-UTR of Gli1 mRNA. Transfection of miR-132 mimic significantly reduced luciferase activity, indicating the targeted regulatory relationship between miR-132 and Gli1 mRNA. Compared with control group, miR-132 expression was decreased and Gli1 level was elevated in glioma tissues, both of which were correlated with the pathological grade. Transfection of miR-132 mimic or si-Gli1 remarkably suppressed the expression of Gli1, Vimentin or Cyclin D1 in U251 cells, up-regulated E-cadherin expression, suppressed cell proliferation and invasion.

CONCLUSIONS: Our data indicated that over-expression of miR-132 could inhibit proliferation or invasion of glioma cells via targeted inhibition of Gli1 expression.

Key Words
MiR-132, Gli1, Glioma, Proliferation, Invasion.

Introduction

Glioma represents a type of common malignant tumor in central nervous system (CNS) and occupies about 60-70% of all intracranial cancers[1]. Glioma is characterized by highest incidence among all intracranial tumors, and is prevalent in individuals with 10-15 and 30-40 years old[2]. Brain glioma is the second cancer for number of deaths, and severely threatens children health[3,4]. Due to features such as high malignancy, potent invasion, and high recurrent or metastatic rate, the survival rate and prognosis in patients with glioma are extremely unfavorable.

Hedgehog signal pathway widely participates in the embryonic development or tissue repair, and can regulate cell proliferation, apoptosis, epithelial mesenchymal transition (EMT), cell migration and invasion[5,6]. Glioma-associated oncogene protein 1 (Gli1) is an important effector of downstream of Hedgehog signal pathway. As a transcriptional factor, Gli1 can be translocated from cytoplasm to nucleus, thus activating Hedgehog signal pathway and mediating transcription and expression of various nuclear target genes, eventually controlling cell proliferation, apoptosis, migration and invasion[7-9]. Potentiation of Gli1 expression and function can abnormally activate Hedgehog signal pathway, and affect occurrence, progression and metastasis of various tumors[10-12]. Multiple studies[13,14] showed significantly elevated Gli1 expression in glioma tumor tissues, closely associated with glioma metastasis.

MicroRNA is a kind of endogenous small molecule non-coding single-stranded RNA with 22-25 nucleotides. Via complementary binding to 3'-untranslated region (3'-UTR) of target gene mRNA, it can regulate target gene expression by degrading or inhibiting translation of mRNA,

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thus participating in the modulation of cell proliferation, differentiation and migration, functioning as an oncogene[15] or tumor suppressor gene[16]. Previous study[17] showed miR-132 expression in glioma tumor tissues was abnormally reduced. This study aims to determine the effect of miR-132 on glioma cell proliferation or invasion.

Patients and Methods

Patients

A total of 51 glioma patients who received treatment in Shandong Provincial Hospital affiliated to Shandong University from January 2016 to December 2016 were recruited in this study. All patients received confirmed diagnosis by pathological examinations. There were 28 males and 23 females, aging between 40 and 72 years (average age = 56.3 ± 14.7 years). Pathological grading showed 17, 22 and 12 cases at stage II, III and IV, respectively. Another cohort of 12 brain samples collected from contusion traumatic surgery was recruited as the control group. There were 6 males and 6 females in the control group, aging from 38 to 69 years (average age = 54.7 ± 12.8 years). No significant differences of age or sex ratio were observed between two groups. The study protocol was approved by the Research Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University, and all patients gave their informed consent before study commencement.

Reagents and Materials

Glioma U251 cell line was purchased from Gefan Cell Bank (Shanghai, China). High-glucose Dulbecco's Modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were acquired from Gibco (Rockville, MD, USA). RNA extraction buffer RNAiso Plus was obtained from TaKaRa (Otsu, Shiga, Japan). Real-time quantitative polymerase chain reaction (qPCR) reagent TransScript quantitive reverse transcript polymerase chain reaction (qRT-PCR) SuperMix was bought from Quanshijin Bio (Beijing, China). miR-NC, miR-132 mimic, and transfection reagent RiboFECT™ CP was purchased from Ruibo Bio (Guangzhou, China). Rabbit anti-E-cadherin, Vimentin and Cyclin D1 antibody were collected from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-Gli1, β-actin and horseradish-peroxidase (HRP) conjugated secondary antibody was purchased from Abcam (Cambridge, MA, USA). Transwell chamber was bought from Corning (Corning, NY, USA). Matrigel was from BD Biosciences (Franklin Lakes, NJ, USA). Dual-luciferase activity assay kit and pMIR luciferase reporter plasmid were obtained from Promega (Madison, WI, USA).

U251 Cell Culture

U251 glioma cells were inoculated in high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS), and were incubated in a humidified chamber with 5% CO_2 at 37°C. Culture medium was changed every 2-3 days, and cells were passed every 3-4 days.

Dual Luciferase Gene Reporter Assay

Using HEK293T cell genome as the template, full-length fragment of 3'-UTR of Gli1 gene was amplified and purified from agarose gel electrophoresis. After dual enzymatic digestion, amplification products were ligated into luciferase reporter gene pMIR plasmid followed by transforming DH5α competent bacteria. Positive clones with correct sequences were selected by sequencing. Those clones with correct sequences were used for cell transfection and further experiments, named as pMIR-Gli1-wt and pMIR-Gli1-mut. RiboFECT™ CP was used to co-transfect pMIR-Gil1-wt (or pMIR-Gil1-mut) and miR-243 mimic (or miR-NC) into HEK293T cells. After 48 h incubation, dual luciferase activity was measured following manual instruction of test kit.

Over-Expression of miR-231 in U251 Cells

U251 cells were divided into two groups and inoculated into 10 cm culture dish. At 50-60% confluence, cells were transfected with miR-NC or miR-132. In brief, 100 µl riboFECT™CP Buffer were used to dilute 5 µl miR-NC or miR-132 mimic followed by 5 min room temperature incubation. 10 µl RiboFECT™ CP reagent were then added for gentle mixture and 0-15 min room temperature incubation. The riboFECT™ CP mixture was added into culture medium for 72 h of continuous incubation. Cells were collected for measuring the efficiency of over-expression.

siRNA Interference of Gli1 in U251 Cells

U251 cells were divided into two groups and inoculated into 10 cm culture dish. Cells were transfected with si-NC or si-Gli1 as above-mentioned. After 72 h of continuous incubation, cells were collected for testing the efficiency of over-expression.

qRT-PCR for Gene Expression

TransScript Green One-Step qRT-PCR SuperMix was used to test the relative expression of target genes using RNA extracted by RNAiso Plus as the template in one-step qRT-PCR. In a 20 μL reaction system, there were 1 μg RNA template, 0.3 μM forward and reverse primers, 10 μL 2 X TransStart Tip Green qPCR SuperMix, 0.4 μL RT Enzyme Mix, 0.4 μL Dye II and distilled water. qRT-PCR conditions were: 45°C for 5 min for reverse transcription, followed by 94°C 30 s, and 40 cycles each containing 94°C 5 s, 60°C 30 s. Gene expression was measured on an ABI ViiA™ 7 Real-time fluorescent PCR cycler.

Western Blot

Total proteins were extracted by sodium dodecyl sulfate (SDS) lysis buffer. After measuring protein purity and concentration, 3 volumes of 4 X protein loading buffer were added and bolt for 5 min. 40 μg samples were loaded and separated in 8-10% separating sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separating gel plus 5% condensing gel. Proteins were transferred into polyvinylidene difluoride (PVDF) membrane by electrophoresis. The membrane was blocked with 5% defatted milk powder for 60 min at room temperature. Primary antibodies (E-cadherin at 1:3000 dilution, Vimentin at 1:3000, Gli1 at 1:2000, Cyclin 1 at 1:3000, and β-actin at 1:10000) were added for incubation at 4°C overnight. The membrane was rinsed in phosphate-buffered saline tween-20 (PBST) for three times, and horseradish-peroxidase (HRP) conjugated secondary antibody (1:30000) was added for 60 min incubation at room temperature. Enhanced chemiluminescence (ECL) approach was used to test the protein expression.

Flow Cytometry for Measuring Cell Proliferation

Transfected cells from all groups were re-suspended and incubated in 10 μM EdU for 2 h. After 48 h of continuous incubation, cells were digested by trypsin and collected. After centrifugation, fixation, and permeabilization, reaction buffer containing Alexa Fluor 488 labels were added for 30 min dark incubation at room temperature. By centrifugation and washing, Beckman Coulter FC500 MCL/MPL flow cytometry (Brea, CA, USA) was used to test cell proliferation.

Transwell Assay for Cell Invasion Potency

100 μL Matrigel were paved on the filter membrane of transwell chamber, which was incubated at 37°C chamber for 30 min for complete polymerization. 500 μL complete culture medium containing 10% fetal bovine serum (FBS) were added into 24-well plate. Transwell chamber was placed into 24-well plate. The upper chamber was filled with 200 μL U251 cells from all transfection groups in serum-free medium. After 48 h of continuous incubation, cells were fixed in methanol and stained with crystal violet. The amount of perforated cells was counted under an inverted microscope.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student *t*-test was used to compare the difference between groups. A statistical significance was defined when $p<0.05$.

Results

miR-132 Targeted and Inhibited Gli1 Expression

Online gene prediction (microRNA.org) showed the existence of complementary binding sites between miR-132 and 3'-UTR of Gli1 mRNA (Figure 1A). Dual luciferase gene reporter assay showed that transfection of miR-132 mimic significantly suppressed the relative luciferase activity in HEK293T cells (Figure 1B), indicating the targeted regulation between miR-132 and Gli1 mRNA.

Down-Regulation of miR-132 and Up-Regulation of Gli1 in Glioma

qRT-PCR showed that, compared with brain contusion tissues without tumor lesion, Gli1 mRNA expression in glioma tissues was significantly elevated (Figure 2A). With more advanced pathological grade, Gli1 expression was increased correspondingly. In addition, miR-132 expression in glioma tissues was significantly reduced. With more advanced TNM stage and pathological grade, its expression was further decreased (Figure 2B). Western blot results also showed significantly higher Gli1 protein expression in glioma tissue compared with control group, and the level was correlated with pathological grade (Figure 2C).

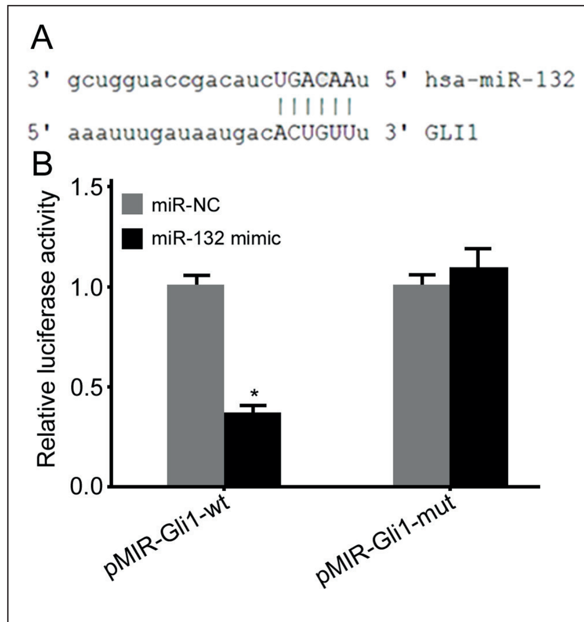

Figure 1. MiR-132 targeted and inhibited Gli1 expression. **A**, Binding sites between miR-132 and 3'-UTR of Gli1 mRNA; **B**, Dual luciferase reporter gene assay. *, $p<0.05$ compared with miR-NC group.

Over-Expression of mi-132 Targeted and Inhibited Gli1 Expression, Suppressed U251 Cell Proliferation or Invasion

Compared with miR-NC transfection group, miR-132 and E-cadherin mRNA expressions were significantly elevated after miR-132 mimic transfection. However, the mRNA levels of Gli1, Vimentin and Cyclin D1 were significantly decreased (Figure 3A). Western blot results showed remarkable increase of E-cadherin with reducing level of Gli1, Vimentin, and Cyclin D1 (Figure 3B). Flow cytometry showed that miR-132 mimic transfection decreased the proliferation potency of U251 cells by 34.5% (Figure 3C). In addition, transwell assay showed that the invasion potency of U251 cells was significantly reduced after the over expression of miR-132 compared with miR-NC transfected cells (Figure 3D).

siRNA Interference of Gli1 Significantly Inhibited the Proliferation and Invasion Potency of U251 Cells

Compared with si-NC transfection group, down regulation of Gli1 significantly caused the elevation of E-cadherin mRNA expression, whereas, the mRNA level of Gli1, Vimentin and Cyclin D1 was significantly decreased (Figure 4A). Western blot showed similar result (Figure 4B). Flow cytometry showed that transfection of si-Gli1 decreased the proliferation potency of U251 cells by 40.6% (Figure 4C) and significantly inhibited invasion potency of U251 cells than that of si-NC transfected cells (Figure 4D).

Discussion

Hedgehog signal pathway is a highly conserved signal pathway closely related with embryonic development, organogenesis and tissue differentiation[5,6]. Hedgehog signal pathway consists of extracellular signal ligand HH, transmembrane protein

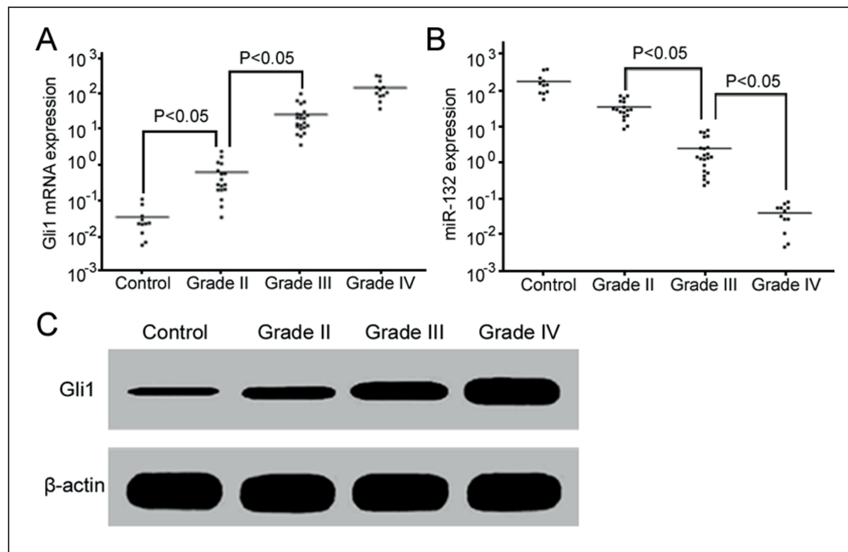

Figure 2. Down-regulation of miR-132 and up-regulation of Gli1. **A**, qRT-PCR for Gli1 mRNA expression; **B**, qRT-PCR for miR-132 expression; **C**, Western blot for Gli1 protein expression.

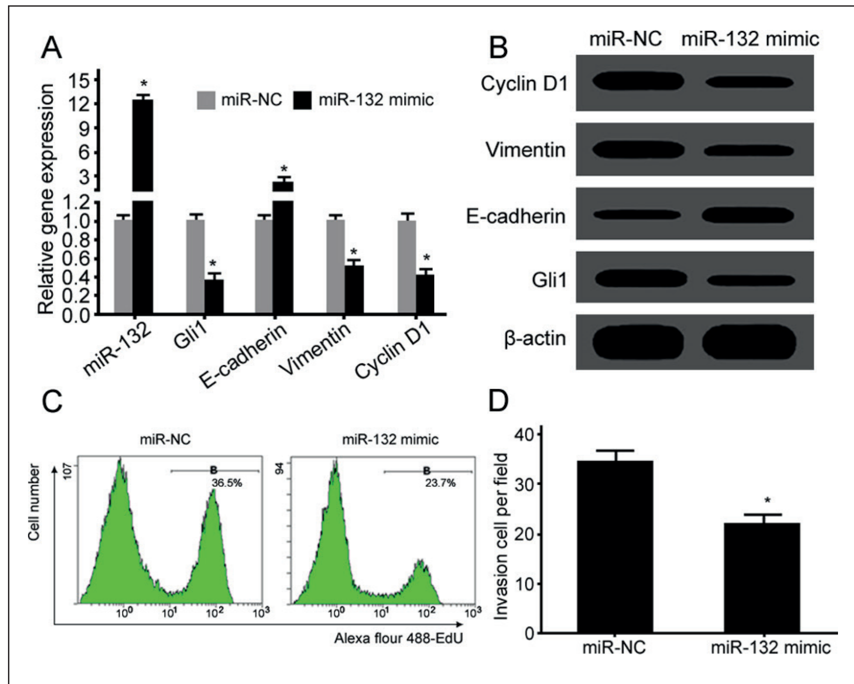

Figure 3. miR-132 over-expression targeted and inhibited Gli1 expression, suppressed U251 cell proliferation or invasion. **A**, qRT-PCR for gene expression; **B**, Western blot for protein expression; **C**, Flow cytometry for cell proliferation; **D**, Transwell for cell invasion. *, $p<0.05$ compared with miR-NC group.

receptor Ptch, an alternative transmembrane protein Smoothened (Smo), intermediate transmission molecule, transcription factor Gli and downstream target genes. When Hedgehog signal pathway is under inactive state, Ptch binds to Smo to suppress its activity, phosphorylates Gli, degrades subsequent ubiquitination, and inhibits the entry into nucleus. The activation of Hedgehog signal pathway eventually participates in the regulation of cell proliferation, migration and malignant transformation leading to tumorigenesis[18]. Human Gli protein family consists of Gli1, Gli2 and Gli3, of

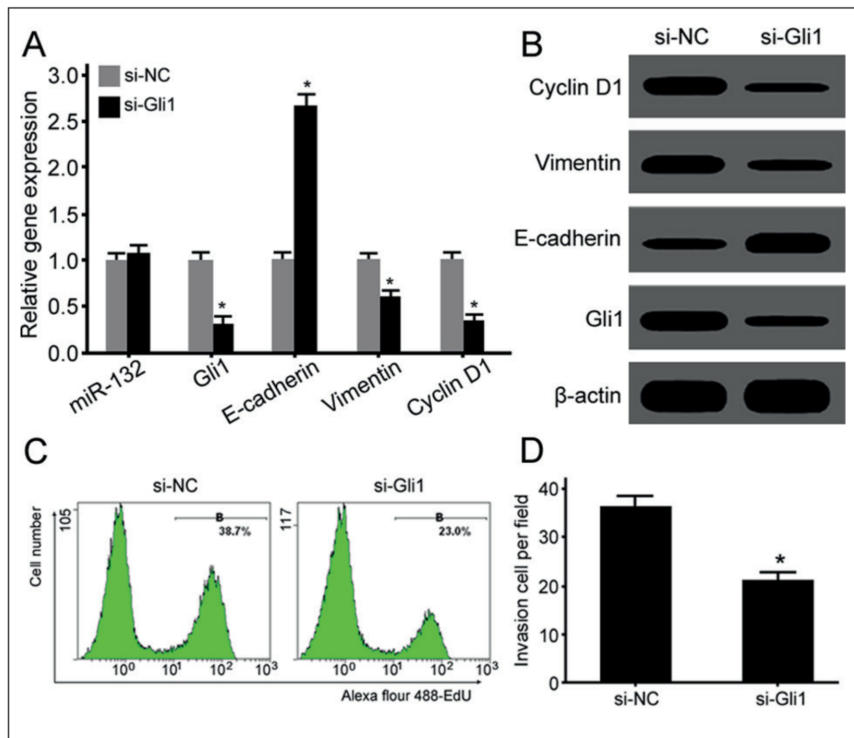

Figure 4. siRNA interference on Gli1 significantly inhibited proliferation or invasion potency of U251 cells. **A**, qRT-PCR for gene expression; **B**, Western blot for protein expression; **C**, Flow cytometry for cell proliferation; **D**, Transwell for cell invasion. *, $p<0.05$ compared with si-NC group.

which Gli1 is the most important one with solely activating function. Abnormal up-regulation of Gli1 indicates over-activation of Hedgehog signal pathway[19]. The abnormal elevation of Gli1 is typically correlated with the onset, progression and prognosis of various tumors including pancreatic carcinoma[20], breast cancer[21] and prostate cancer[22]. Various studies showed significant increase of Gli1 expression in glioma tissues[13,14], which is closely correlated with glioma metastasis.

Chen et al[17] also showed miR-132 expression was abnormally decreased in glioma tissues. Bioinformatics analysis showed targeted relationship between miR-132 and 3'-UTR of Gli1 gene. Dual luciferase gene reporter assay validated that transfection of miR-132 mimic significantly depressed the relative luciferase activity inside HEK293T cells, demonstrating targeted regulation between miR-132 and Gli1 gene. Compared with brain contusion tissues without tumor lesion, miR-132 expression glioma tissues were significantly lower, whereas Gli1 expression level was remarkably elevated. Moreover, the expression changes of miR-132 and Gli1 were correlated with the pathological grade, indicating possible roles of miR-132 down-regulation in potentiating Gli1 expression and subsequently facilitating glioma pathogenesis and progression. Chen et al[17] showed that, compared with normal brain tissues, miR-132 expression glioma tissues were significantly reduced, with even lower level in patients at terminal stage in clinic. Wang et al[23] also showed abnormally decreasing level of miR-132 expression in glioma tissues compared with normal brain tissues, and miR-132 expression in grade III-IV stage glioma was even reduced compared to that in grade I-II tumors. Liao et al[13] showed significantly higher Gli1 expression in glioma tissues than normal brain tissues and higher Gli1 expression in glioma cell lines U251 or U87 compared with normal human astrocytes. These results were consistent with our observations showing lower Gli1 expression in glioma tissues compared with normal brain tissues. Our results showed that transfection of miR-132 mimic remarkably elevated miR-132 expression, inhibited U251 cell proliferation and invasion potency. Moreover, siRNA interference targeting Gli1 expression exerted similar effects to miR-132 over-expression, showing that proliferation or invasion potency of U251 cells was impeded. Multiple studies showed that, as the transcriptional regulatory factor of Hedgehog signal pathway, Gli1 could facilitate the expression of downstream target genes Cyclin D1 and Bcl-2, further facilitating cell proliferation and antagonizing cell apoptosis[24,25]. Therefore, this investigation further observed whether miR-132 or Gli1 may affect Cyclin D1 expression to mediate glioma cell proliferation. Our data showed that transfection of miR-132 mimic or si-Gli1 suppressed U251 cell proliferation via targeted inhibition of Gli1 and downstream gene Cyclin D1 expressions. Chen et al[17] showed that miR-132 up-regulation could inhibit the proliferation or migration of glioma cell line U87, and decrease resistance against chemotherapy, via targeted inhibition on TTK proteinase. Li et al[26] showed that over-expression of miR-132 inhibited the expression of SIRT1 or SREBP-1c, suppressed glioma cells U251 or U87 proliferation, migration or invasion, facilitated cell apoptosis, as well as inhibited the growth velocity of glioma cells inside animal body and tumor formation potency. Wang et al[23] showed that miR-132 could regulate MMP-16 expression and inhibit the migration or invasion potency of glioma cells A172, SHG44 and U87. Geng et al[27] also showed that in glioma cells U87 and U251, a targeted regulation relationship existed between miR-132 and PEA-15 gene, and over-expression of miR-132 could significantly inhibit the proliferation or migration potency of U87 and U251 cells but increase the apoptosis. In this study, up-regulation of miR-132 expression was also found to reduce the proliferation, invasion or other malignant biological features of glioma cells, supporting those previous studies. We found that down-regulation of Gli1 expression could inhibit cell proliferation or invasion, consistently with Liao et al[13] and Wang et al[14] findings. We showed that regulation of Gli1 and downstream target gene Cyclin D1 expression might be a mechanism underlying the inhibition of glioma cell proliferation by miR-132. However, a large scale of clinical trial requires to be performed to evaluate the practical effect of miR-132 in the treatment of glioma. Also, the combined use of suppression of miR-616[28], miR-21[29], miR-130b[30], for instance, along with the over expression of miR-132, should be tested in the inhibition of glioma cell invasion.

Conclusions

We showed that over-expression of miR-132 could decrease the proliferation or invasion potency of glioma cells via the inhibition of GLi1 expression, which lays fundamental support for the future therapy of glioma.

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Conflict of Interest:

The Authors declare that they have no conflict of interests.

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