MiR-130b can suppress proliferation of glioma cells through targeting PTEN to regulate AKT pathway

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Summary

Purpose: To study the mechanism of action of micro ribonucleic acid (miR)-130b in the proliferation and apoptosis of glioma cells, and to determine whether it regulates the target gene phosphatase and tensin homolog deleted on chromosome ten (PTEN).

Methods: The effects of miR-130b silencing on the proliferation and apoptosis of LN229 cells were detected using cell counting kit-8 (CCK-8) assay, colony formation assay and flow cytometry. The changes in the mRNA level of PTEN after miR-130b silencing were determined through quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The effects of miR-130b on protein kinase B (AKT) signaling pathway-related proteins were determined through Western blotting.

Results: Compared with that in normal human astrocytes, the expression of miR-130b was significantly up-regulated in the three kinds of glioma cell lines (p<0.05). Silencing of miR-130b reduced the proliferation (p<0.05) and the colony formation of LN229 cells (p<0.05), and obviously increased their apoptosis (p<0.05), suggesting that silenced miR-130b is a growth inhibitor of glioma cells in vitro. The luciferase reporter assay confirmed that miR-130b directly binds to the 3'-untranslated region (3'UTR) of PTEN to suppress its expression. After transfection with the miR-130b inhibitor, both mRNA and protein expressions of PTEN were up-regulated (p<0.05). Moreover, after silencing miR-130b, the phosphorylation of AKT was remarkably inhibited, while the cancer suppressor gene p27 was up-regulated.

Conclusions: The carcinogenic effect of miR-130b in glioma was clarified in this study. Silencing of miR-130b may inhibit the AKT signaling pathway through up-regulating PTEN, thereby suppressing the proliferation of glioma cells.

Key words: miR-130b, glioma, PTEN, AKT signaling pathway

Introduction

Among all human malignant brain tumors, glioma occupies the highest proportion, and its most malignant type is glioblastoma multiforme (GBM, WHO class IV) [1,2]. Although glioma patients have been treated with advanced treatment means, such as radioactive rays and chemotherapy, the prognosis remains poor, and the median survival time is less than 16 months [3,4]. The rapid growth of glioma cells caused by genomic instability and genetic disorder is a typical feature of glioma [5]. In addition, there are limited treatment strategies for glioblastoma due to the incomplete understanding of its pathogenesis. Therefore, studies are needed to clarify the basic mechanism of GBM development, and find novel and effective treatment strategies for this disease.

Micro ribonucleic acids (miRs) are a class of small endogenous non-coding RNAs with about 22 nucleotides in length, which play extensive roles in embryonic development, cell proliferation, differentiation, apoptosis, cell cycle progression, angiogenesis and other biological processes [6]. Studies
have demonstrated that the abnormal expression of specific miRs is closely related to uncontrolled proliferation, cell cycle arrest, invasion, migration and resistance to chemotherapy [7,8]. MiR-130b is an important member of the miR-150 family. Previous research has shown that miR-130b downregulates the cell cycle inhibitor p21Waf1/Cip1 in breast tissues, thereby inhibiting apoptosis of mammary epithelial cells and promoting tumor growth [9]. In addition, Dong et al [10] studied and found that the mutated p53 gene induces epithelial-mesenchymal transition (EMT) through regulating the miR-130b/ZEB1 axis in endometrial cancer, thereby triggering tumor development. It has also been observed that the expression of miR-130b is significantly lower in endometrial cancer tissues than in normal tissues, and patients with a high expression level of miR-130b have a longer survival time according to the long-term follow-up of patients with endometrial cancer [10]. These studies suggest that miR-130b can exert an anti-tumor effect or tumor-promoting effect based on the type of tumors. Moreover, it was concluded in a study of Malzkorn et al [11] that the expression of miR-130b is significantly increased with the development of glioma from the second stage to the fourth stage in four patients. However, the function and mechanism of miR-150b in glioma need to be clarified. Therefore, this study aimed to explore the role of miR-130b in glioma.

Methods

Materials

The following materials were used in this study: Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), apoptosis assay kit (Sigma, St. Louis, MO, USA), Pierce bicinechonic acid (BCA) protein concentration assay kit (Thermo, Waltham, MA, USA), TRizol reagent and Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan), SYBR Premix Dimer Eraser Kit (TaKaRa, Tokyo, Japan), pmirGLO plasmids, reverse transcription kit, and dual luciferase assay system (Promega, Madison, WI, USA), phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphorylated-protein kinase B (p-AKT), p21 and β-actin antibodies (Cell Signaling Technology, Danvers, MA, USA), flow cytometer (BD, Franklin Lakes, NJ, USA), and 7900HT Fast real-time fluorescence quantitative polymerase chain reaction (qPCR) system (Thermo, Waltham, MA, USA).

Cell culture and transfection

The normal human astrocyte (NHA) cell line, and glioma LN229, U251 and B2-17 cell lines used were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in 2018 (Shanghai, China). All cell lines were cultured in the DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator with 5% CO₂ at 37°C. The negative control (NC), miR-130b mimic or inhibitor and siRNA targeting PTEN (si-PTEN) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). LN229 cells (1×10⁵) were inoculated into 6-well plates and transfected according to the instructions of the Lipofectamine 3000 reagent. After transfection for 6 h, the medium was discarded, and the cells were cultured with the complete medium in the incubator, and then collected at the corresponding time points.

QPCR analysis

At 48 h after transfection, the total RNA was extracted from LN229 cells using TRizol reagent, and 1 μg of total RNA was reversely transcribed into cDNA using the reverse transcription kit, followed by qPCR on the 7900HT Fast system using the SYBR Premix Dimer Eraser Kit and oligonucleotide primers as follows: miR-130b F: 5’-CTC GGC AGT CAG GGG GAG T-3’, R: 5’-GTC TCC GCT GCT TTC AGA CGA T-3’. PTEN F: 5’-TTT GAA GCC CTC TTT GAT GCT GTC C-3’, R: 5’-GAA GCC TCC TTT GAT GCT GTC C-3’. GAPDH F: 5’-GAA GCC CTC TTT GAT GCT GTC C-3’, GAPDH R: 5’-TGT GGG CAT CAA TGG ATT TGG-3’. The PCR conditions were as follows: 95°C for 3 min, amplification for 40 cycles (95°C for 12 s, 62°C for 1 min, 72°C for 1 min), and extension at 72°C for 2 min. The relative expression levels of genes were calculated using 2^ΔΔCq. All PCRs were repeated 3 times.

CCK-8 assay

After digestion with trypsin, about 2×10⁴ cells were inoculated into 96-well plates. At 24, 48, and 72 h after inoculation, CCK-8 working solution was added, followed by incubation in the incubator for 5 h. Then the optical density (OD) in each group was measured at 450 nm using a microplate reader. The experiment was repeated 3 times.

Colony formation assay

The transfected LN229 cells were inoculated into the 6-well plates (300 cells/well) and cultured for 2 weeks for colony formation. The DMEM medium was replaced every 3 days. Then, the colonies were fixed in methanol for 5 min and stained with 1.0% crystal violet dye for 30 s. Finally, the number of visible colonies was counted.

Analysis of apoptosis

At 48 h after transfection, the cells were collected and stained with Annexin V-FITC and propidium iodide (PI). The apoptosis rate of transfected LN229 cells was detected using a flow cytometer.

Western blotting

After transfection, the cells were collected, washed with cold phosphate buffered saline, and lysed with ra-
dioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentration in the lysate was determined using the Pierce bicinchoninic acid (BCA) protein assay kit. After separation via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with 5% skim milk powder for 1 h, and incubated with PTEN (1:1000), p-AKT (1:1000), p21 (1:1000) and β-actin (1:1000) primary antibodies at 4°C for 12 h. After the membrane was washed, the protein was incubated again with corresponding secondary antibodies at room temperature for 60 min, followed by image development using the electrochemiluminescence (ECL) system.

Luciferase activity assay

Based on the search results in TargetScan, the PTEN 3’-untranslated region (3’UTR) sequences predicted or mutant sequences with predicted target sites were synthesized and inserted into the pmirGLO plasmids (pmirGLO-PTEN-3’UTR-wt and pmirGLO-PTEN-3’UTR-mut). In the reporter gene assay, LN229 cells were inoculated into 24-well plates, and transfected with pmirGLO-PTEN-3’UTR-wt or pmirGLO-PTEN-3’UTR-mut and miR-130b mimic or NC using Lipofectamine 3000. At 48 h after transfection, the cells were harvested and analyzed using the dual-luciferase reporter assay system.

Statistics

In all statistical analyses, two-tailed Student’s t-test in SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was used for the analysis of intergroup differences. The experiment was repeated 3 times. P<0.05 suggested that the difference was statistically significant.

![Figure 1](image1.png)

**Figure 1.** Expression level of miR-130b in NHA and glioma LN229, U251 and B2-17 cells detected using qRT-PCR (*p<0.05).**

![Figure 2](image2.png)

**Figure 2.** Effects of miR-130b silencing on proliferation and colony formation of LN229 cells. **A:** Expression level of miR-130b detected via qRT-PCR. **B:** Proliferation of LN229 cells in miR-130b inhibitor group and NC group detected using CCK-8 assay. **C:** Clone ability of LN229 cells in miR-130b inhibitor group and NC group detected using colony formation assay (*p<0.05) (magnification: 40×).
Results

Overexpression of miR-130b in glioma cells

The expression level of miR-130b was detected using qRT-PCR in NHA and glioma LN229, U251 and B2-17 cells. It was found that the expression of miR-130b was significantly up-regulated in the three kinds of glioma cell lines compared with that in NHA (p<0.05, Figure 1), indicating that miR-130b is overexpressed in glioma cells, and it may have a carcinogenic effect in occasion.

Effects of miR-130b silencing on proliferation and colony formation of LN229 cells

To clarify the effect of miR-130b on glioma, LN229 cells were transfected with the miR-130b inhibitor to reduce the expression of miR-130b (Figure 2A). Then the proliferation and colony formation of transfected LN229 cells with the miR-130b inhibitor were detected via CCK-8 assay and colony formation assay. It was observed that silencing of miR-130b reduced the proliferation and the colony formation of LN229 cells (Figure 2B & 2C), suggesting that silenced miR-130b is a growth inhibitor of glioma cells in vitro.

Effect of miR-130b silencing on apoptosis of LN229 cells

Flow cytometry was performed to determine the apoptosis of LN229 cells in miR-130b inhibitor group and NC group. The results revealed that silencing of miR-130b obviously increased the apoptosis of LN229 cells (Figure 3).

PTEN was a direct target gene for miR-130b

To study the biological mechanism of miR-130b, its target gene was predicted, and it was found that PTEN containing the binding sites of miR-130b was a potential target gene for miR-130b (Figure 4A). Luciferase reporter assay further confirmed that miR-130b directly binds to the PTEN 3'UTR to suppress its expression (Figure 4B).

Effect of miR-130b on AKT signaling pathway

LN229 cells were transiently transfected with the miR-130b inhibitor to silence the expression of miR-130b, and then the total RNA and protein were extracted at 48 h after transfection. The RNA and protein expressions of PTEN were measured. The results manifested that both mRNA and protein expressions of PTEN were up-regulated in cells transfected with the miR-130b inhibitor compared with that in cells transfected with NC (Figure 5A & 5B). Moreover, after silencing of miR-130b, the phosphorylation of AKT was remarkably inhibited, while the cancer suppressor gene p27 was up-reg-
The above findings demonstrate that silencing of miR-130b may inhibit the AKT signaling pathway through up-regulating PTEN.

To confirm the roles of miR-130b and PTEN in the proliferation of glioma cells, LN229 cells were co-transfected with the miR-130b inhibitor and si-PTEN. The results of CCK-8 assay showed that the proliferation of cells co-transfected with the miR-130b inhibitor and si-PTEN was remarkably enhanced compared with that of cells transfected with the miR-130b inhibitor alone (Figure 5C), demonstrating that silencing endogenous PTEN can reverse the effect of miR-130b silencing, and confirming that miR-130b silencing inhibits the proliferation of glioma cells via up-regulating PTEN expression.

**Discussion**

The most advanced comprehensive treatment is currently available, but the prognosis of GBM patients remains poor [12]. The anti-angiogenesis therapy is an effective treatment for glioma. However, the median survival time of GBM patients is about 14.6 months from the date of diagnosis, and the 5-year survival rate is <3% [13]. Therefore, with the rapid development of molecular biological techniques for GBM in recent years, better understanding the molecular mechanism of malignant progression of GBM and finding new therapies for GBM have become the main direction of research [14].

It has been confirmed that a single miR molecule can act on multiple or even hundreds of target genes, and several different miRs may regulate the same gene, so miRs and their targeting proteins form a complex regulatory network, thereby regulating cell growth and development, and tumorigenesis [15,16]. The miR-130 family includes miR-130a and miR-130b, and both are co-localized on chromosome 22 (10 kb apart), and they can have tandem expression in cells, suggesting that they may possess similar biological functions [17]. Tu et al [18] detected the increased expression of miR-130b in human hepatocellular
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carcinoma (HCC) and liver cancer cell lines, which is associated with poor prognosis of liver cancer patients. It has also been proved that inhibiting the expression of miR-130b up-regulates its target gene via PPAR-γ, thereby suppressing invasion and migration of HCC. In addition, according to a study of Zhao et al [19], the expression of miR-130b declines in pancreatic cancer, and the relation between down-regulation of miR-130b and poor prognosis of patients with pancreatic cancer was also determined. Studies have found that overexpression of miR-130b inhibits the proliferation and invasion of pancreatic cancer cells through down-regulating the target gene STAT3. However, the expression pattern of miR-130b and its potential specific mechanism in the pathogenesis of glioma have not been fully clarified yet. Therefore, this study aimed to explore the mechanism of action of miR-130b in the proliferation and apoptosis of glioma cells and determine whether it regulates the target gene PTEN. In this study, it was found via qRT-PCR that compared with that in normal human astrocytes, the expression of miR-130b was significantly up-regulated in the three glioma cell lines (LN229, U251 and B2-17). Silencing of miR-130b in LN229 cells inhibited cell proliferation and induced apoptosis. The above findings indicate that miR-130b acts as an oncogene in glioma cells.

PTEN is a cancer suppressor gene located on chromosome 10q23.3, with dual specificity [20]. As a kind of lipid phosphatase, PTEN up-regulates the cancer suppressor gene p27 and inhibits Cyclin D1 to regulate the cell cycle, thus leading to cell cycle arrest in G0/G1 phase and preventing cells from entering S phase [21]. In this study, transfection of LN229 cells with the miR-150b inhibitor could promote the expression of target gene PTEN and reduce the phosphorylation of AKT. In glioma cells transfected with the miR-150b inhibitor, the expression of p27 was increased. Besides, silencing of PTEN reversed the inhibition on cell proliferation.

Conclusions

In conclusion, the carcinogenic effect of miR-130b in glioma was clarified in this study, which may be associated with the regulation of the PTEN/AKT signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

References

17. Lee EK, Lee MJ, Abdelmohsen K et al. miR-130 suppresses adipogenesis by inhibiting peroxisome prolif-


