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ORIGINAL ARTICLE

The effect of miR-21 on SWOZ2 glioma cells and its biological mechanism

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Summary

Purpose: *To investigate the role of micro RNA-21 (miR-21) in human glioma cells and its potential disease-causing mechanism.*

Methods: *jetPRIME was used to transfect the miR-21-mimics and its negative control into SWOZ2 human glioma cells. Real-time fluorescence quantitative PCR assay was used to measure differences in the expression of miR-21 in SWOZ2 glioma cells, SWOZ2-miR-21-mimics cells, and control cells. Cell counting kit-8 assay was used to measure the activity of SWOZ2 glioma cells and SWOZ2-miR-21-mimics cells, and Western blot was used to measure PTEN, p-Akt, and P-glycoprotein (P-gp).*

Results: *The level of miR-21 in SWOZ2-miR-21-mimics cells was significantly higher than in SWOZ2 cells and the negative control group. Compared with SWOZ2 cells, the expression of PTEN protein in SWOZ2-miR-21 cells decreased significantly, and the expression of p-Akt and P-gp protein were significantly increased. Compared with SWOZ2 cells and the negative control group, the proliferation rate of SWOZ2-miR-21-mimics cells was significantly increased ($p<0.05$).The rate of apoptosis as determined by flow cytometry showed that the number of apoptotic SWOZ2-miR-21-mimics cells decreased significantly ($p<0.05$). Transwell assay found that the invasive ability of SWOZ2-miR-21-mimics cells increased significantly, suggesting that miR-21 can mediate the biological functions of SWOZ2 cells by inhibiting the expression of PTEN.*

Conclusion: *miR-21 may regulate the proliferation and apoptosis of human glioma cells by downregulating the expression of the PTEN protein, and miR-21 may represent a potential therapeutic target for the treatment of glioma.*

Key words: *glioma; miRNA-21*

Introduction

Glioma is the most common primary malignant tumor of the central nervous system, with a high rate of mortality and disability. It severely affects the quality of life of patients and their families [1,2]. Glioma accounts for roughly 50% of intracranial tumors, and its pathogenesis is related to many factors, such as genetics, ionizing radiation, the biological environment, environmental pollution, and infection. However, the exact molecular mechanism of its pathogenesis remains unclear [3,4]. Clinical diagnosis of glioma is usually made in the middle and late stage, and the prognosis is poor. Therefore, it is of great significance to explore the molecular mechanisms of its pathogenesis and related markers, as well as its early diagnosis and clinical treatment [5].

miRNAs are non-coding RNA species that function in regulating the expression of target genes. A large number of studies has proven that they are closely related to the occurrence and development of malignant tumors [4]. In malignant tumors, some miRNAs have abnormal expression,

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which plays the role of oncogenes or tumor suppressor genes, having great impact on the incidence of malignant tumors and disease progression [5]. However, this is not clear in studies of glioma [6-8].

In the present study, we further explored the biological role of miR-21 in glioma, to provide a theoretical basis for the possible identification of therapeutic targets.

Methods

Materials and reagents

The following materials and reagents were used: Cell Counting Kit-8 (CCK-8) (Beyotime Biotechnology, Beijing); Trizol reagent (Invitrogen, USA); Prime Script TM reverse transcription kit, SYBR Premix Ex TaqTM; TaqTM fluorescent quantitative PCR Kit (TaKaRa, Japan); miR-21 and U6snRNA Bulge-LoopTM miRNA qRT-PCR PrimerSet (Ruibo company, Guangzhou, China); miR-21mimics and miRNA mimics negative control (Shanghai Zimmer company); jet PRIMETM Transfection Reagent (Polyplus Transfection); Chromosome 10 homology deleted phosphatase and tension protein, and phosphorylated protein kinase B (p-Akt) rabbit anti-human monoclonal antibody (Cell Signaling, USA); P-glycoprotein (P-gp) mouse anti-human monoclonal antibody; 0.45 μm cellulose nitrate membrane (Millipore, MM, USA); β-actin mouse anti-human monoclonal antibody; horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, HRP-labeled goat anti-mouse IgG (Protein Tech Group, Rosemont); BCA protein quantitative Kit (Beyotime Biotechnology, Beijing); ECL substrate luminescence detection kit (Thermo Fisher Scientific, USA).

Instruments and equipment

CO_2 gas incubator (Thermo, USA); Inverted phase contrast microscope (Olympus, Japan); ELx800 automatic enzyme standard instrument (Bio-Tek, USA); Mini Opticon Real-Time PCR System, vertical electrophoresis apparatus, and electric transfer apparatus (Bio-Rad, USA).

Cell culture

The human brain glioma cell line, SOWZ2, was constructed in the laboratory and stored frozen [3,4]. SOWZ2 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in an incubator set to 37°C and 5% CO_2 with saturated humidity. For cell digestion, 0.25% trypsin and 0.02% EDTA were used.

RFQ-PCR to detect the expression of miR-21 in cells

Total RNA was extracted from cells using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and measured by spectrophotometry. The A260/A280 value was confirmed to be between 1.8 and 2.1 for cDNA synthesis. Briefly, 1 ml Trizol per 5×10^5 cells was added, prior to adding 0.2 ml of chloroform per 1 ml Trizol. Samples were mixed vigorously by hand and allowed to stand for 2–3 min at room temperature. The mixture was then centrifuged at 10,000 g for 10 min at 4°C. The upper clear phase was transferred to a fresh tube and 0.5 ml isopropanol per 1 ml of the clear phase was added, mixed vigorously by rapid shaking, and left to stand for 10 min. The precipitated RNA was collected by centrifugation at 10,000 g for 10 min at 4°C and then the supernatant was carefully decanted/pipetted. The RNA precipitate was washed once with 70% ethanol, dissolved in 25 μl RNase-free water, and then stored at −80°C. The concentration of Recombinant DNase I (RNase-free) (Takara Biotechnology Co., Ltd., Dalian, China) used to treat RNA samples was 5 U/μl.

cDNA was synthesized using the HiFi-MMLV cDNA kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) and qPCR was conducted using the UltraSYBR Mixture (Beijing ComWin Biotech Co., Ltd.). Briefly, 5 μg purified RNA were mixed with Primer Mix, dNTP Mix, DTT, RT-buffer, HiFi-MMLV, and RNase-free water using a pulled pipette (total volume, 20 μl). All qRT-PCR reactions were run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mixture was then incubated at 42°C for 30–50 min followed by 85°C for 5 min. The RT products were quickly centrifuged and stored at −20°C. No cDNA was used as negative control. To amplify hsa-miR-21-3p cDNA, specific RT primers were used based on its sequence, and the U6 RT primer was the same as the U6 reverse PCR primer. The hsa-miR-21-3p RT primer was 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAAAG-3'. PCR primers were as follows: Forward, 5'-GGCACCACACCTTCTACAAT-3' and reverse, 5'-GTGGTGGTGAAGCTGTAGCC-3' for the β-actin gene; forward, 5'-CAGTGGGGAACTCTGACTCG-3' and reverse, 5'-GTGCCTGGTGCTCTCTTACC-3' for the HOTAIR gene; forward, 5'-CGGTCAGTGCATCACAGAA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3' for hsa-miR-21-3p; and forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3' for U6. All primers were synthesized by GenScript (Nanjing, China). The relative fold changes of mRNA expression level were calculated using the $2^{-\Delta\Delta Cq}$ method.

Transfection miR-21 mimics

SWOZ2 cells in the logarithmic growth phase were digested, counted, and placed in each well of a 6-well plate at a concentration of 5×10^5 cells/well. Cells in the SWOZ2-miR-21-mimics group were treated with 30 nmol/l miR-21-mimics, 200 μl jet PRIMETM Buffer, and 4 μl jet PRIMETM Reagent. Cells in the negative

control group were treated with miRNA-mimics negative control at the same concentration as miR-21 mimics, 200 µl jet PRIMETM Buffer, and 4 µl jet PRIMETM Reagent (Control group or SWOZ2-miR-control mimics group). The two groups of cells were cultured in RPMI-1640 supplemented with 5% FBS. After 24 hrs, the culture solution was replaced with RPMI-1640 containing 10% FBS, and the transfection protocol was performed in strict accordance with the instructions. After 48 hrs transfection, total cellular RNA was extracted by RFQ-PCR to measure the expression of miR-21.Western blot was used to measure protein expression in the cells of each group. The survival rate of cells was measured by CCK-8.

MTT assay

The viability of SWOZ2 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were trypsinized and seeded at a density of 1×10^4 cells/well in 96-well plates immediately after miRNA transfection. Next, 10 µl MTT solution (5 mg/ml) was added and the plates were incubated for an additional 4 hrs at 37°C. Following removal of the medium, formazan crystals were dissolved in 150 µl dimethyl sulfoxide. The absorbance of MTT formazan was measured at 550 nm using a SpectraMax M3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Experiments were repeated 3 times using 6 wells for each condition to ensure the reproducibility of results.

Flow cytometry (FCM)

Cells were individually harvested at 0, 24, 48, and 72 hrs after transfection, fixed in 70% ethanol, and stained with propidium iodide (PI) (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China) containing RNase A (1 mg/ml; Takara Biotechnology Co., Ltd.) for 30 min at 37°C. Subsequently, 500 µl of cells were filtered through 200-µm mesh sieves, and the cell cycle profiles were assayed using a Guava easyCyte 8 Flow Cytometer (EMD Millipore, Billerica, MA, USA).

Matrigel invasion assay

Transwell inserts (diameter 6.5 mm) with pore size 8 µM (Corning Incorporated, Corning, NY, USA) were coated with Matrigel (100 µg/well; BD Biosciences, San Jose, CA, USA) and placed in wells of 24-well culture plates. Following transfection with miRNA for 48 hrs, 1×10^4 cells were transferred to the top of the invasion chambers in serum-free DMEM, while DMEM containing 20% fetal calf serum (FCS) was added to the lower chambers. After 24 hrs of incubation at 37°C, non-invasive cells were removed with a cotton swab, and the invading cells were fixed with 4% paraformaldehyde for 15 min, stained with Giemsa for 20 min at room temperature, and observed under an ECLIPSE Ti S microscope (Nikon,Tokyo,Japan). Experiments were independently repeated three times.

Statistics

All statistical analyses were performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean±standard deviation of experiments performed in triplicate. GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) was used for graphing. Statistical analysis of data from other experiments was performed using one-way ANOVA, the Student-Newman-Keuls test was performed for multiple comparisons, and unpaired Student's t-tests for comparisons between groups. $P<0.05$ was considered statistically significant.

Results

The expression of miR-21 in SWOZ2 cells in each group

The expression of miR-21 in SWOZ2 and SWOZ2-miR-21-mimics cells was measured by RFQ-PCR. The results showed that the expression in SWOZ2 and SWOZ2-miR-21-mimic were 1.021±0.079 and 2.077±0.238, respectively. The expression of miR-21 in SWOZ2-miR-21-mimics cells was significantly higher than that of miR-control and mimics cells in the SWOZ2 group ($p<0.01$; Figure 1).

Western blot-mediated detection of the expression of PTEN protein

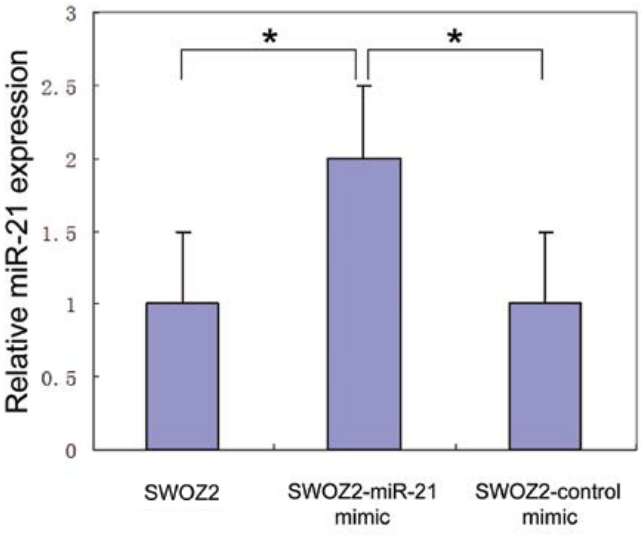

Figure 1. The expressions of SWOZ2-miR-21-mimic and SWOZ2-control-mimic were 1.021 ± 0.079 and 2.077 ± 0.238, respectively. The expression of miR-21 in SWOZ2-miR-21-mimic cells was significantly higher than that of SWOZ2 cells and SWOZ2-control-mimic, and the difference was statistically significant (*$p<0.01$).

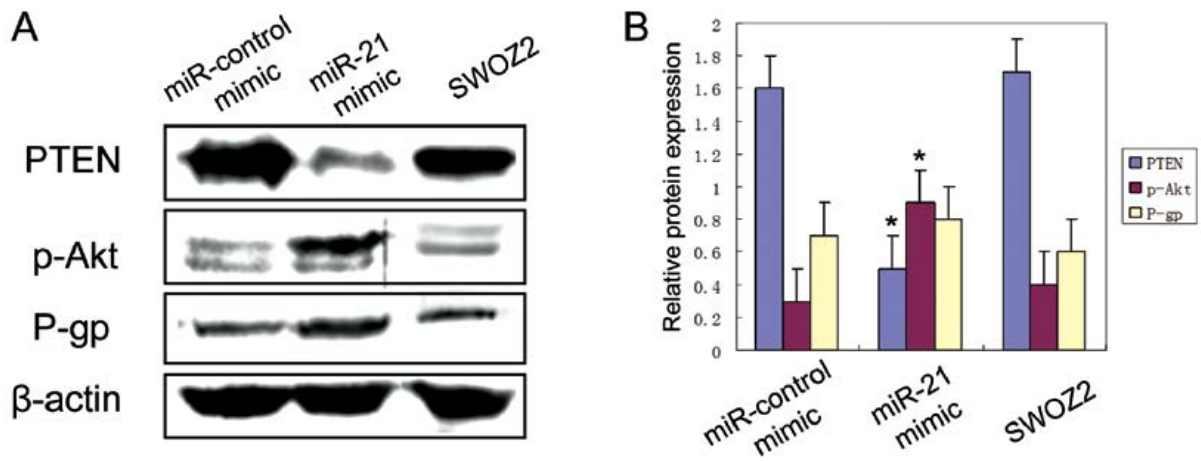

Figure 2. In the miR-21-mimics group, the level of PTEN expression was significantly decreased ($p<0.05$), while the p-Akt level was significantly increased **(A)**, compared with other groups ($p<0.05$) **(B)**. (*Compared with miR-control group, $p<0.05$).

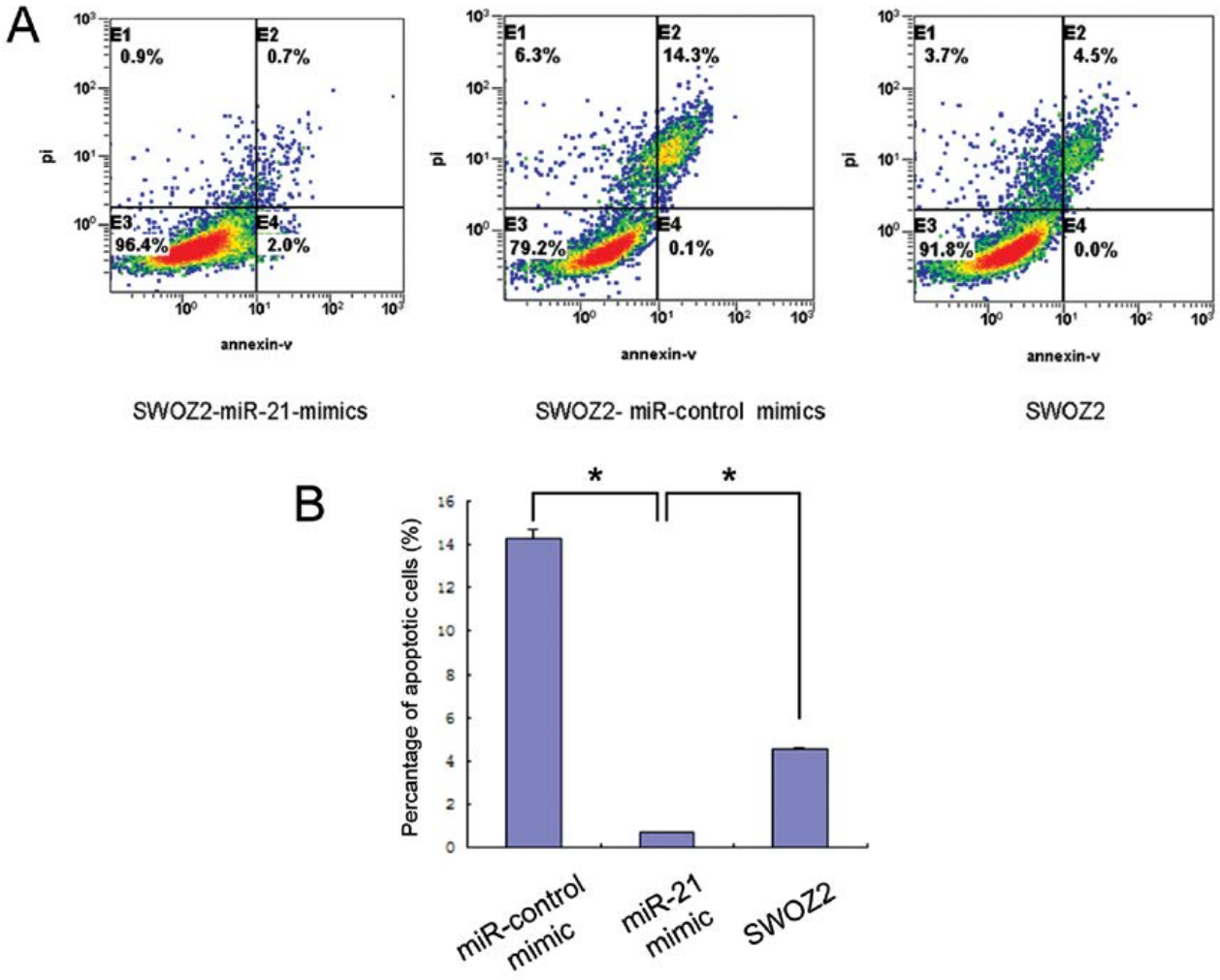

Figure 3. (A) After continuous culture for 48 hrs, Annexin V-FITC/PI double staining was used to measure apoptosis of SWOZ2 cells. The results showed that, over time, the number of apoptotic SWOZ2 cells increased gradually ($p<0.05$). **(B)** miR-21 showed a significant inhibitory effect on cell apoptosis ($p<0.05$).

β-actin was used as the internal reference. The expression of PTEN, p-Akt, and P-gp were measured by Western blot analysis. In the miR-21 mimics group, the level of PTEN was significantly decreased, while p-Akt was significantly increased compared with the other groups ($p<0.05$; Figure 2).

The effect of miR-21 on apoptosis of SWOZ2 human glioma cells

To understand the effect of miR-21 on the apoptosis of SWOZ2 cells, Annexin V-FITC/PI double staining and flow cytometry were used to measure apoptosis of SWOZ2 cells before and after transfection. After continuous culture for 48 hrs, Annexin V-FITC/PI double staining was used to measure the apoptosis of SWOZ2 cells. The results showed that, over time, the number of apoptotic SWOZ2 cells increased gradually ($p<0.05$). miR-21 showed a significant inhibitory effect on cell apoptosis (Figure 3).

The invasive ability of cells as determined by Transwell assay

We used Transwell assay to determine the invasive ability of cells. Compared with the other two groups, cell invasion in the miR-21-mimics group was significantly increased ($p<0.05$;Figure 4).

Discussion

Many studies have indicated that a variety of signaling molecules and signaling pathways may play a role in glioma [9-11].

A recent study found that small, noncoding miRNAs which are involved in post transcriptional regulation play an important role in cell differentiation, tumorigenesis, proliferation, apoptosis, embryonic development, invasion ability, and drug resistance [12]. Since the discovery of Lin-4 in 1993, the miRNA gene sequence database (http://www.mirbase.org/Release18:November2011) currently contains 18,226 mature miRNA species, which includes those from animals, plants, and viruses. A total of 1527 of them are expressed in humans. Different from the fully complementary function of siRNA, the interaction between miRNA and target mRNA is fully complementary or partially complementary. Additionally, multiple miRNAs can act on the same mRNA [13]. MiRNA species may represent key nodes of gene regulatory networks, and they may represent new targets for tumor therapy [14].

In gliomas, 17 miRNAs including miR-21, miR-125b, miR-221, miR-222, and miR-10b were over-expressed, while the expression of 33 miRNAs including miR-7, miR-181a/b/c, miR-124, miR-137 and miR-128 were decreased. A total of 33 miRNAs were downregulated [15,16] in gliomas. miR-21 is located in the fragile region of chromosome 17q23.2, and has the activity of a proto-oncogene. miR-21 is highly expressed in many malignant tumors, such as breast cancer, liver cancer, and brain glioma. Additionally, its expression is positively correlated with the malignant grade of the tumor. In gliomas with different grades, the expression of miR-21 can be used as an independent prognostic indicator of the tumor [17]. It has been confirmed that knock-down of miR-21 in GBM cells inhibited cell growth, increased apoptosis, and decreased invasive ability. As a result, the tumor was inhibited.

In our study, we found that the expression of miR-21 in SWOZ2-miR-21-mimics cells was significantly higher than that of SWOZ2 cells and the negative control group. Compared with SWOZ2 cells, the expression of PTEN protein in SWOZ2-miR-21 cells decreased significantly, and the expression of p-Akt and P-gp protein was significantly increased. Compared with SWOZ2 cells and the negative control group, the proliferation rate of SWOZ2-miR-21-mimics cells was significantly increased ($p<0.05$). Flow cytometric detection of apoptosis showed that the number of apoptotic SWOZ2-miR-21-mimics cells decreased significantly ($p<0.05$). Transwell-mediated detection of

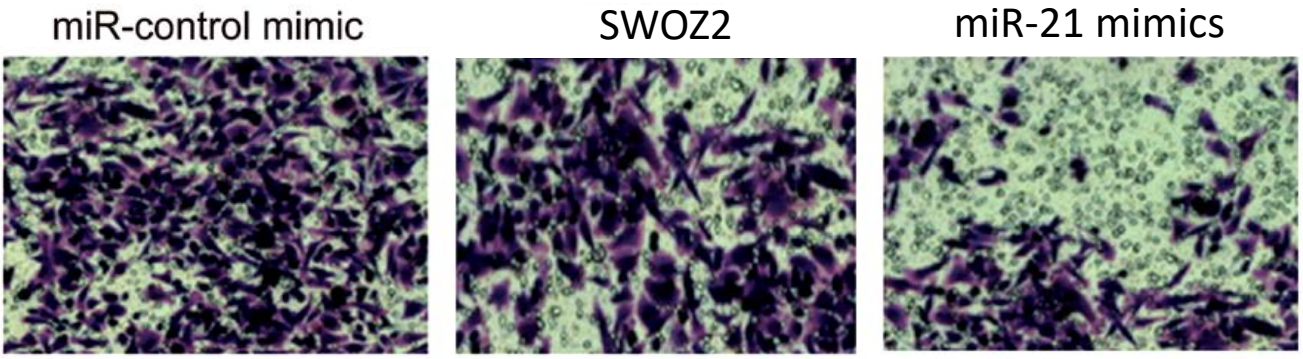

Figure 4. Compared with the other two groups, cell invasion in the miR-21-mimics group was significantly increased ($p<0.05$).

cell invasion showed that in SWOZ2-miR-21-mimics cells, the cell number increased significantly, indicating that miR-21 can inhibit SWOZ2 biological function mediated by the expression of PTEN.

miR-21 may regulate the expression of certain proteins through transcription. Some studies found that the tumor suppressor PTEN is a direct target of miR-21 in several tumors, such as hepatocellular carcinoma and breast cancer. In gliomas, especially high-grade gliomas, the PTEN gene is often mutated or deleted. The expression of PTEN is often down-regulated or missing. The PTEN protein is a negative regulator of the PI3K/Akt pathway. Therefore, the drug resistance of gliomas can be increased after PTEN overexpression. In our study, we found that in the miR-21-mimics group, the number of apoptotic SWOZ2 cells decreased significantly, and cell invasion was significantly increased, while following transfection of miR-21, the expression of PTEN was significantly reduced. Therefore, we believe that PTEN may be a downstream target of miR-21. The interaction of miR-21 with the 3'-UTR of PTEN can reduce PTEN gene transcription and translation, thereby regulating apoptosis and invasion of glioma cells. However, this conclusion requires verification by micro-RNA array and other bioinformatic analyses.

miR-21 may regulate the proliferation and apoptosis of human glioma cells by down-regulating the expression of PTEN protein, and may represent a potential therapeutic target for the treatment of glioma.

Conflict of interests

The authors declare no confict of interests.

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