Effects of mir-128a on the invasion and proliferation of glioma U251 cells

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Abstract. Effects of mir-128a on the proliferation and migration of human glioma U251 cells were explored. The constructed mir-128a-shRNA lentivirus vector (infection group) and scramble shRNA (interference group) were transfected into glioma U251 cells, and uninfected U251 cells as control group. The expression level of mir-128a, the ability of proliferation, invasion, apoptosis and migration of cells in each group were detected by RT-qPCR, MTT assay, Transwell migration in vitro, cell wound scratch assay and TUNEL cell apoptosis assay. The expression level of mir-128a in U251 cells of infection group was significantly higher than that in U251 cells of interference group (P<0.05). The expression level of mir-128a in U251 cells of control group was significantly lower than that in U251 cells of infection group (P<0.05). The OD values of infection and control group were lower than that of interference group at 6, 12, 24, 48 and 72 h, and the OD values of infection were lower than that of control group at 6, 12, 24, 48 and 72 h (P<0.05). Compared with infection and control group, the number of membrane-penetrating cells in U251 cells of interference group increased significantly (P<0.05). The apoptosis rate of U251 cells of infection and control group was significantly higher than that of interference group, and the apoptosis rate of infection was significantly higher than that of control group (P<0.05). The migration distance of U251 cells of infection and interference group was significantly larger than that of control group (P<0.05). The migration distance of U251 cells of interference group was significantly larger than that of infection group (P<0.05). mir-128a may play a role similar to anti-oncogene in glioma, inhibiting the ability of proliferation, invasion and migration of glioma cells, and promoting the apoptosis of glioma cells.

Introduction

Glioma is the most common primary malignant tumor of the intracranial tumors, accounting for approximately 45% of all intracranial tumors. The prognosis of glioma patients is generally poor, and the higher the malignant degree is, the worse the prognosis of glioma patients is (1,2). It has been reported (3) that the median survival time of patients with grade IV polyglioblastoma is only one year with less than 5% of 5-year survival rate, which is one of the malignant tumors with the highest human mortality. The treatment of glioma is mainly surgery, supplemented by radiotherapy and chemotherapy (4). Despite the continuous development of medical technology in recent years, the improvement of survival rate of glioma patients is still limited. Many studies have reported that the high mortality rate of glioma is closely related to the excessive proliferation and invasion of tumor cells (5,6).

miRNAs is widely expressed in eukaryotic organism, regulating cell proliferation, differentiation and apoptosis, while abnormal changes in miRNAs biosynthesis are involved in many pathophysiological processes (7,8). Many studies have reported that miRNAs are closely related to biological behavior such as proliferation and invasion of tumor cells (9,10). Guo et al (11) reported that the increased expression level of mir-128a in hepatocellular carcinoma (HCC) could promote the proliferation of HCC cells. However, in the study of Yamada et al (12), it was found that mir-128a could increase the anti-apoptosis ability of Jurkat cells in leukemia patients. Therefore, we speculate that mir-128a plays different roles in different tumors, but there are few studies on the relationship between mir-128a and the biological behavior of glioma cells.

The purpose of this study is to analyze the effects of mir-128a on the biological behavior of glioma U251 cells by regulating the expression level of mir-128a to provide experimental and theoretical basis for clinical treatment, to prolong the patient’s life span and to improve the patient’s quality of life.

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Table I. Primer sequences.

| Genes     | Sequence                                      |
|-----------|-----------------------------------------------|
| mir-128a  | 5'-ACACTCCAGCTGGGTACAGTGAACCG-3' 5'-CCCCAGGTTATGAGCATAATGCAAAAT-3' |
| GAPDH     | 5'-CGGAGTCACCGGATTTGGGTGTAT-3' 5'-AGGCTTTCTCCATGGTGGTGGAAGAC-3' |

Materials and methods

Cell source. **Human glioma U251 cells** (cat. no. CC-Y1526), purchased from Shanghai Enzyme Research Biotechnology Co., Ltd. (Shanghai, China), were cultured in DMEM (containing 10% fetal bovine serum) culture medium (Beijing North Tongzheng Biotechnology Development Co., Ltd., Beijing, China). The culture conditions of human glioma U251 cells were 37˚C and 5% CO₂, and the construction and synthesis of mir-128a expression vector and scramble shRNA were constructed by Shanghai GenePharma Biology Co. (Shanghai, China). The constructed mir-128a-shRNA lentivirus vector (infection group) and scramble shRNA (interference group) were cultured together with human glioma U251 cells digested with trypsin in DMEM (containing 10% fetal bovine serum) culture medium, and then transfected after cultured at culture medium with 37˚C 5% CO₂ for 48 h. The specific steps referred to the kit instructions and other experimental tests were performed after transfection. The Liposome 2000 transfection kit was purchased from Shanghai Bayley Biotechnology Co., Ltd. (Shanghai, China). A group of U251 cells, which was not infected, was used as control group.

The study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China).

Extraction of total miRNA from cells. TRIzol was used to extract and collect (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) the total RNA of human glioma U251 cells according to the instructions. The concentration and purity of extracted RNA were analyzed by Micro ultraviolet spectrophotometer GeneQuant1300/100D (GE Medical System Trade Germany) the total RNA of human glioma U251 cells was extracted RNA, and the RNA specimens of A260/A280 between 1.8 and 2.0 were considered to meet the test standard. The integrity of RNA was analyzed by 3% agarose gel electrophoresis (gel electrophoresis set was purchased from Shanghai Jingke Chemical Technology Co., Ltd., Shanghai, China).

Reaction of mir-128a to RT-qPCR. The total RNA extracted above was synthesized of cDNA by reverse transcription according to the instructions in the TaqMan MicroRNA reverse transcription kit [Synevir Technology (China) Co., Ltd., Shenzhen, China]. The cDNA amplification reaction system was 10 µl, 1.0 µl for oligo DT primer, 1.0 µl for dNTP mixture, 2 µg for total RNA, 1 µl for Taq DNA polymerase, and non-ribonuclease distilled water added to 10 µl. Reverse transcription reaction: 37˚C for 45 min and 65˚C for 5 min. The reaction system was 50 µl, 2 µl for cDNA template, 32.5 µl for SYBR-Green Mix (Guangzhou Dongsheng Biotechnology Co., Ltd.), 0.5 µl for upstream primer and 0.5 µl for downstream primer, and double distilled water added to 50 µl. PCR amplification: 3 min after pre-denaturation at 95˚C, 30 sec for denaturation at 95˚C, 30 sec for annealing at 55˚C, 60 sec for extension at 72˚C, 30 cycles, and 5 min for extension at 72˚C after the completion of cycle. GAPDH was used as the internal parameter of the reaction. All the samples were repeated three times and the result was analyzed by 2^ΔΔCt (13) method. Primer sequences are shown in Table I.

Proliferation in vitro of human glioma U251 cells detected by MTT assay. The human glioma U251 cells were prepared into single cell suspension and 96-well cell culture plate was used for cell routine inoculation culture. The cells were taken out at 6 h, and 20 µl MTT (5 mg/ml) were added. The supernatant containing impurity was sucked out at 37˚C for 4 h, and then the dimethylsulfoxide was added and placed on a horizontal shaking bed for 15 min. Finally, the absorbance at 570 nm wavelength was determined by enzyme linked immunosorbent assay (ELISA). The above steps were repeated in the experiment at 12, 24, 48 and 72 h, respectively. MTT test kit was purchased from Shanghai Lianmai Bioengineering Co., Ltd., Shanghai, China.

Transwell invasion in vitro. The prepared U251 cell suspension was inoculated into the Transwell chamber and the number of cells passed through was detected after two weeks. Three parallel trials were conducted simultaneously. The Transwell chamber was purchased from Shanghai Yuanzi Biotechnology Co., Ltd., Shanghai, China.

TUNEL cell apoptosis assay. U251 cells were cultured for 48 h, approximately 5x10⁵/ml, were fixed at room temperature with 4% neutral formaldehyde for 10 min and the excess liquid was removed and washed twice with PBS for 5 min each time. PBSs containing 2% hydrogen peroxide were treated at room temperature for 5 min and the excess liquid was removed and washed twice with PBS for 5 min each time. TUNEL assay solution (50 µl) was added to each well (the ratio of TdT enzyme to fluorescent labeling solution was 1:24), and incubated at 37˚C for 60 min. The cells were dyed according to the instructions provided by TUNEL kit (Shanghai Rong Wei Da Industrial Co., Ltd., Shanghai, China). The cell was sealed with antifade mounting medium and stored at 2-8˚C. The number of TUNEL-positive cells in 5 fields of vision under the 400-fold microscope was counted by image analysis software (Image-Pro Plus 5.0), and the cumulative optical density value was used to indicate the total number of TUNEL-positive cells. The assay was repeat three times.

Cell migration ability detected by wound scratch assay. The cells transfected with each group for 48 h were inoculated...
to a six-well plate, and three groups of repeat wells were set up. When the cell fusion reached approximately 90%, 20 µl was used perpendicular to the 6-well culture plate and drawn according to the pre-prepared horizontal line. PBS was used to wash the plate three times, and 1% FBS DMEM medium was used for continuous cultivation. This was repeated three times with imaging measurement. Cell imaging system Phase-contrast microscope (EVOS® FL Cell Imaging System), Thermo Fisher Scientific, Inc., Waltham, MA, USA.

Statistical analysis. Using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). The comparison of ratio was tested with χ². The measurement data were expressed as mean ± standard deviation, the comparison of groups was analyzed by ANOVA and the differences between two groups were detected and analyzed by LSD. The differences were statistically significant (P<0.05).

Results

Analysis of mir-128a detection in U251 cells by RT-qPCR. The result of RT-qPCR showed that the expression level of mir-128a in U251 cells of infection group was significantly higher than that of interference group (P<0.05). The expression level of mir-128a in HA1800 cells of normal control group was significantly lower than that of infection group (P<0.05), but higher than that of mir-128a in U251 cells of interference group (P<0.05). Figure 1.

Cell proliferation of U251 cells detected by MTT assay. The result of MTT assay showed that the OD values of infection and normal control group were lower than that of interference group at 6, 12, 24, 48 and 72 h, and the OD values of infection were lower than that of normal control group at 6, 12, 24, 48 and 72 h. The cell proliferation ability of infection and normal control group was lower than that of interference group, and the cell proliferation ability of U251 cells of infection group was lower than that of HA1800 cells of normal control group (P<0.05). The differences were statistically significant (P<0.05). *P<0.05, more infection group; *P<0.05, more normal control group.

Invasion of U251 cells detected by Transwell migration in vitro. The result of Transwell showed that compared with infection group, the number of membrane-penetrating cells in U251 cells of interference group increased significantly (P<0.05). The number of membrane-penetrating cells in U251 cells of control group was higher than that of infection group. *P<0.05. Figure 2.

Invasion of U251 cells and HA1800 cells detected by Transwell migration in vitro. The result of Transwell showed that compared with infection group, the number of membrane-penetrating cells in U251 cells of interference group increased significantly (P<0.05). The number of membrane-penetrating cells in U251 cells of control group was higher than that of infection group. *P<0.05. Figure 3.
the number of membrane-penetrating cells in U251 cells of infection group increased significantly (P<0.05). The number of membrane-penetrating cells in U251 cells of control group was 123.72±11.45, which was higher than that of infection group (P<0.05), but lower than that of interference group (P<0.05) (Fig. 3).

Apoptosis of U251 cells detected by TUNEL cell apoptosis assay. The result of apoptosis of U251 cells detected by TUNEL cell apoptosis assay showed that the apoptosis rate of infection and control group (38.47±1.26%, 28.32±1.23%) was significantly higher than that of interference group (11.88±1.11%). The apoptosis rate of infection group was significantly higher than that of control group, and the differences were statistically significant (P<0.05) (Fig. 4).

Migration ability of U251 cells in cell wound scratch assay. Through observing the width of the wound scratch under an inverted optical microscope, it could be found that the migration distance of U251 cells of infection group and interference group was significantly larger than that of U251 cells of control group, and the difference was statistically significant (P<0.05). The migration distance of U251 cells of interference group was significantly larger than that of U251 cells of infection group, and the difference was statistically significant (P<0.05) (Fig. 5).

Discussion

The occurrence and development of tumor is a pathological process where multi-step and multi-molecular are involved, and its most important biological features are the ability of almost infinite proliferation and extremely high ability of invasion and metastasis of malignant tumor cells (14,15). The main reason for the high recurrence rate and low survival rate of glioma patients is the invasiveness and metastasis of glioma cells (16,17). Therefore, it is of great significance to explore the related molecular mechanism of glioma cell proliferation and invasion, to provide experimental and theoretical reference for inhibiting the excessive proliferation and invasion of glioma cells, to increase the survival rate of patients and to improve their quality of life.

In this study, mir-128a-shRNA lentivirus vector was constructed to upregulate the expression level of mir-128a in human glioma cell U251, and scramble shRNA was constructed to interfere with the expression of mir-128a in human glioma cell U251. The result of comparison with the expression level of mir-128a in uninfected U251 cells showed that the expression level of mir-128a in U251 cells of infection group was significantly higher than that in U251 cells of interference group, and the expression level of mir-128a in U251 cells of control group was significantly lower than that in U251 cells of infection group, but higher than that in U251 cells of interference group. The expression vector was successfully transfected into glioma U251 cells and expressed successfully, which was consistent with the previous expectation. In this study, the result of biological behavior detection of glioma U251 cells showed that upregulation the expression level of mir-128a could inhibit the ability of proliferation, invasion and migration of glioma U251 cells, and promote apoptosis level of glioma U251 cells. Therefore, we conclude that mir-128a could inhibit the malignant biological behavior of glioma cells.

Nie et al (18) reported that upregulation of mir-128a could inhibit the proliferation ability of glioma U87 cells. Venkataraman et al (19) found that mir-128a target regulated (79.13±12.04), the number of membrane-penetrating cells (177.58±13.49) in U251 cells of interference group increased significantly (P<0.05). The number of membrane-penetrating cells in U251 cells of control group was 123.72±11.45, which was higher than that of interference group (P<0.05), but lower than that of control group (P<0.05) (Fig. 3).
Bmi-1, increased the level of intracellular reactive oxygen species, and promoted the senescence of neural tubular tumor cells, thereby inhibiting tumor proliferation. From these results we can infer that mir-128a may play a role as anti-oncogene in central nervous system tumors, but the mechanism of action of mir-128a in glioma cells needs to be further verified. The Bmi-1 and reactive oxygen species level is a very good direction and Bmi-1 gene is one of the core members of the PcG family. Bmi-1 is highly expressed in tumor cells, which makes tumor cells regenerate into cancer stem cells (19). Nevertheless, De Luca et al (20) reported that lowering the level of mir-128a could induce the increase of Lin28a expression and LIN28 could regulate the self-renewal of stem cells and improve bone marrow differentiation disorder in patients with acute myeloid leukemia. Guo et al (11) found that miR-128a was upregulated in hepatocellular carcinoma and promoted the proliferation of hepatocellular carcinoma cells by targeting RND3 and RND3 was a member of the Rnd subgroup of the Rho family of GTP enzymes and regulated the tissue of actin cytoskeleton through the response of extracellular growth factors. From their results we can find that mir-128a plays a role similar to oncogene in hepatocellular carcinoma and acute myeloid leukemia. Therefore, we speculate that mir-128a may inhibit the biological behavior of tumors in the central nervous system, such as glioma, thus promoting the biological behavior of hepatocellular carcinoma, leukemia and other organic cancers as well as blood cancer.

Ye et al (21) found that U-87MG glioblastoma cells exposed to X-ray radiation could decrease the expression level of mir-128a, induce the upregulation of the expression level of Bmi-1, and then lead to the decrease of reactive oxygen species in cells, which would cause the cells to escape from aging and death. This may also be one of the reasons why the radiotherapy effects of glioma patients is not satisfactory, and we will further verify the effects of mir-128a on the radiotherapy effects of glioma cells in the future studies. This study also investigated cell lines, so more clinical studies are needed to prove the results of this study.

Collectively, mir-128a may play a role similar to anti-oncogene in glioma, inhibiting the ability of proliferation, invasion and migration of glioma cells, and promoting apoptosis of glioma cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GH drafted the manuscript. GH and WF were mainly devoted to extraction of total miRNA from cells. GH and NL were responsible for transwell invasion in vitro. CL performed RT-qPCR. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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