MIR-129 INHIBITS GLIOMA CELL PROLIFERATION AND PROMOTES AUTOPHAGY AND APOPTOSIS BY TARGETING NOTCH-1

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ABSTRACT

Objective: *To analyze the inhibitory effect of miR-129 on glioma cell proliferation, autophagy, and apoptosis by targeting Notch-1.*

Methods: *Human glioma cell line U251 was cultured and subcultured, then divided into a control group, a miR-129 overexpression group, and a miR-129 inhibition group. U251 cells were transfected with a pE2F7 overexpression vector and an interference vector, and the blank control group was treated with a blank lentivirus vector. MTT was used to detect the cell growth curve; flow cytometry was used to detect the apoptosis rate; real-time PCR was used to detect the expression of LC3-II/LC3-I, p62, and Notch-1 mRNA; and Western blot was used to detect the protein expression of LC3-II/LC3-I, p62, and Notch-1.*

Results: *The proliferation of the three groups increased with time. The proliferation of the miR-129 overexpression group was significantly lower than that of the control group at day 3 through day 7, and the cell proliferation in the miR-129 inhibition group was significantly higher than that of the control group (P<0.05). The apoptosis rate of the miR-129 group was significantly higher than that of the control group, and the apoptosis rate of the miR-129 inhibition group was significantly lower than that of the control group (P<0.05). The expression levels of LC3 - II/LC3 - I protein and mRNA in the miR-129 group were significantly higher than those in the control group; p62 protein and mRNA expression levels were significantly lower in the miR-129 group than they were in the control group; and p62 protein and mRNA expression levels in the miR-129 group were significantly lower than they were in the control group (P<0.05). The expression levels of Notch-1 protein and mRNA in the miR-129 group were significantly lower than those in the control group, and the expression levels of Notch-1 protein and mRNA in the miR-129 group were significantly higher than those in the control group (P<0.05).*

Conclusion: *miR-129 can inhibit the proliferation of glioma cells, increase the apoptosis rate of glioma cells, and promote autophagy by regulating the expression of autophagy-related proteins. The mechanism may be related to miR-129 regulating Notch-1.*

Keywords: *miR-129, notch-1, inhibition, glioma cells, proliferation, autophagy, apoptosis.*

DOI: 10.19193/0393-6384_2022_1_22

Received *March 15, 2020;* ***Accepted*** *October 20, 2020*

Introduction

A glioma is a malignant tumor originating from the mesenchymal tissue of the neuroectoderm. It is the most common primary malignant tumor in the brain, accounting for 40%-50% of all brain tumors[1]. At present, surgery, radiotherapy, and chemotherapy are the main methods for the treatment of gliomas, but the clinical effects are modest, the patient's life cycle is shortened, and their prognosis is often poor[2]. Therefore, studying the pathogenesis of gliomas and finding effective and safe treatments are a focus of research by scholars in the medical field both at home and abroad. miRNA is a non-coding molecular RNA that exists in eukaryotes.

Many studies have shown that miRNAs play an important role in the proliferation, apoptosis, aging, and differentiation of gliomas. This RNA can even regulate the autophagy and drug resistance of gliomas[3]. Among them, Shouwei et al.[4] found that

the expression of miR-129 in glioma tissues and cells was significantly low. Exogenous overexpression of miR-129 can even inhibit the activity of glioma cells, but the related mechanism is not clear.

This experiment takes the human glioma cell line U251 as the object of observation and aims to analyze the effect of miR-129 on the proliferation, autophagy, and apoptosis of glioma cells and the mechanisms by which it has these effects.

Materials and methods

General information

The human glioma cell line U251 was selected. The cells were provided by Beijing Taize Jiaye Technology Development Co., Ltd.

Methods

Cell culture and passage

The human glioma cell line U251 was cultured in a high-sugar DMEM medium at 37°C, 5% CO_2 concentration, and saturated humidity. The cells were taken out of the cell cryopreservation tube and quickly placed in a 37°C water bath to ensure the cells in the cryopreservation tube melted quickly. The cells in the cryopreservation tube were transferred to a 2 ml centrifuge tube and centrifuged at 800 rpm for 4 minutes. The supernatant was discarded, and then 1 ml of growth medium was added to resuspend the cell pellet. It was transferred to a 10 cm cell culture dish and 9 ml of growth medium was added. It was placed in a 37°C CO_2 incubator for cultivation. The passage was carried out when the cell confluence reached 80-90%, 2-3 days after cell resuscitation. The growth medium was removed, and the membrane enzyme and D'Hanks solution were preheated to room temperature.

The cell culture dish was taken out of the CO_2 incubator and 1 ml of D'Hanks solution was added; 1 ml membrane enzyme was also added. It was observed under an inverted microscope. After the cells retracted and became round, the membrane enzymes were quickly aspirated, and 3 ml of complete medium was added to terminate the digestion. The cell suspension was added to a new petri dish and placed in an incubator for culture.

Cell transfection

The cells were digested and counted using the passaging method and then prepared into a single cell suspension, and the concentration was adjusted to 6×10^4 cells/ml. In a 96-well plate, 200 μL of cell suspension was added to each well and they were placed in a 37°C cell incubator for culture. The next day, transfection was performed when the cell confluence reached 50% under a microscope. U251 cells were transfected with a pE2F7 overexpression vector and an interference vector, and a blank control group was set up, which used a blank lentiviral vector. Each group had 6 repeating holes. Other tests were carried out 48 hours after transfection.

Observation index

MTT detects cell growth curve

The cells were collected, digested, centrifuged, and counted. Using a density of 2000 cells per well, a cell-medium mixture was prepared according to the counting results. 100 μL was added to each well, and 3 multiple wells were set for each group of cells. Continuous plank 6d. It was replaced with a fresh medium and protected from light. 20 μL of 5 mg/mL MTT reagent was added to each well. It was placed in a 37°C incubator and incubated for 4 hours. The culture medium in each well was carefully aspirated, and then 150 μL of DMSO was added. The culture plate was placed on a shaker and shaken at low speed for 10 minutes to fully dissolve the precipitate. The absorbance value was detected with a microplate reader at a wavelength of 570 nm. The cell growth curve was calculated and drawn based on the results.

Flow cytometry to detect cell apoptosis

The cells were collected, digested, and centrifuged, and each tube was washed twice with 2 mL of PBS. Four 1.5 mL EP tubes were used. A cell suspension containing 1.0×10^6 cells was added to each tube. It was centrifuged at 1000 rpm for 5 minutes, and 3000 μL of Binding Buffer was added to resuspend the cells and stain. 5 μL of FITC dye solution was added to each tube of cells and incubated at 4°C for 10 minutes. IOBL PI staining solution was added, and the tubes were incubated at 4° C for 5 minutes. The apoptosis rate was calculated on the computer.

Detection of LC3-II/LC3-I, p62, Notch-1 mRNA expression by real-time quantitative PCR

When the cell line was 80% confluent in a 6-well plate, 700 μL of QIAzol reagent was added to each well, and they were incubated at room temperature for 5 minutes. 140 μL of chloroform was added to every 700 μL of QIAzol reagent.

The wells were incubated at room temperature for 3 minutes and centrifuged for 10 minutes. The colorless upper aqueous phase was transferred to a new EP tube and 1.4 times the volume of absolute ethanol was added. It was pipetted into the RNeasy Mini filter tube and centrifuged at 4°C and 12000 rpm for 30 minutes. Then, 700 μL of RWT solution was added to the filter tube and it was centrifuged at 12000 rpm for 30 minutes at room temperature. Next, 500 μL of RPE solution was added to the filter tube and centrifuged at 12000×g for 15 minutes at room temperature. The RNA pellet was air-dried for 5 minutes, and then 30 μL of RNase-free water was added and the solutions were centrifuged at 12000×g for 15 minutes at room temperature. Then, 2 μL of each sample RNA solution were taken and a micro-ultraviolet spectrophotometer was used to determine the RNA concentration. Real-time quantitative reverse transcription PCR was conducted to obtain relative expression.

Western blot detection of LC3-II/LC3-I, p62, Notch-1 protein expression

An appropriate amount of protein lysate was added at the ratio of 1 μL of PMSF to 1 ml of RIPA. The prepared protein lysate was placed on ice. After discarding the culture medium, it was washed twice with D'Hanks solution.

An appropriate volume of lysate was added, and care was taken to cover the cell surface with lysate. It was placed on ice to react for a few minutes, then centrifuged at 12000 rpm for 10 minutes, before the supernatant was transferred to a new EP tube. The protein solution was mixed with 5X loading buffer, and it became a protein sample after being boiled in water for 10 minutes. The BCA quantitative method was used to calculate the concentration of the sample to be tested. Prepare separation gel and concentrated gel, electrophoresis, transfer, block, incubate the primary antibody, wash the primary antibody, incubate the secondary antibody, wash the secondary antibody, ECL color development.

The exposed film was scanned into the computer for grayscale analysis.

Statistical analyses

This experiment used SPSS 20.0 software for statistical analysis of all collected data. All measurement data were represented by ($\bar{x}\pm s$), and comparison between groups was via t-test. Count data was represented by (%), and an X^2 test was used. The result was statistically significant at P<0.05.

Results

Comparison of cell proliferation in each group

The cell proliferation increased over time in each of the three groups. The cell proliferation of the miR-129 overexpression group was significantly lower than that of the control group at day 3, day 5, and day 7, and the cell proliferation of the miR-129 inhibited group was significantly higher than that of the control group. There was statistical significance (P<0.05). These results are shown in Table 1.

Group	1	3	5	7	Cathepsin K
Control group	1.95±0.14	2.85±0.21	3.79±0.26	4.76±0.21	1.59±0.62
Overexpression of miR-129 group	1.65±0.11[a]	2.38±0.14[a]	2.68±0.18[a]	2.98±0.25[a]	0.99±0.53
Inhibition of miR-129 group	2.84±0.16[a]	3.52±0.25[a]	4.56±0.31[a]	0.31±0.35[a]	0.99±0.53
F	401.080	156.050	273.300	1313.830	2.942
P	<0.001	<0.001	<0.001	<0.001	0.006

Table 1: Comparison of cell proliferation in each group.
[a]p<0.05, compared with the control group.

Comparison of the apoptosis rate of the three groups

The apoptosis rate of the miR-129 overexpression group was significantly higher than that of the control group, and the apoptosis rate of the miR-129 inhibition group was significantly lower than that of the control group. The difference was statistically significant (P<0.05). These results are shown in Table 2.

Grouping	Apoptosis rate
Control group	0.24±0.12
Overexpression of miR-129 group	0.45±0.25[a]
Inhibition of miR-129 group	0.13±0.05[a]
F	19.970
P	<0.001

Table 2: Comparison of the apoptosis rate of the three groups.
[a]p<0.05, compared with the control group.

Comparison of autophagy-related protein and mRNA expression levels in three groups of glioma cells

The expression levels of LC3-II/LC3-I protein and mRNA in the cells in the miR-129 overexpression group were significantly higher than those of the control group, and the expression levels

of p62 protein and mRNA were significantly lower than those of the control group. The expression levels of LC3-II/LC3-I protein and mRNA in the miR-129 inhibition group were significantly lower than those in the control group, and the expression levels of p62 protein and mRNA were significantly lower than those in the control group. The difference was statistically significant (P<0.05). These results are shown in Table 3.

Grouping	LC3-II/LC3-I	P63
Control group	1.26±0.16	0.89±0.11
Overexpression of miR-129 group	3.46±0.22[a]	0.68±0.08[a]
Inhibition of miR-129 group	0.88±0.08[a]	1.16±0.15[a]
F	1447.860	84.730
P	<0.001	<0.001

Table 3: Comparison of autophagy-related protein and mRNA expression levels in three groups of glioma cells. *[a]p<0.05, compared with the control group.*

Comparison of the expression levels of Notch-1 protein and mRNA in each group

The expression levels of Notch-1 protein and mRNA in the miR-129 overexpression group were significantly lower than those in the control group, and the expression levels of Notch-1 protein and mRNA in the miR-129 inhibition group were significantly higher than those in the control group. The difference was statistically significant (P<0.05). These results are shown in Table 4.

Grouping	Notch-1
Control group	0.85±0.22
Overexpression of miR-129 group	0.48±0.15[a]
Inhibition of miR-129 group	0.98±0.21[a]
F	35.110
P	<0.001

Table 4: Comparison of the expression levels of Notch-1 protein and mRNA in the three groups of glioma cells. *[a]p<0.05, compared with the control group.*

Discussion

Gliomas exhibit local invasive growth and rapid growth, which makes it difficult to judge the boundary with normal brain tissue. It is difficult to completely remove the glioma via surgery, and the remaining tumor cells are highly invasive, so the probability of recurrence after surgery is relatively high[5]. Average survival for patients with high-grade glioma is only about 15 months, which seriously endangers their life and health[6]. Previous studies have shown that controlling the proliferation and anti-apoptosis of gliomas is the key to treating gliomas[7]. How to control and delay the growth of glioma cells to avoid poor prognosis has become the clinical synthesis of many neurosurgeons' research efforts and is directing treatment of glioma. miRNA participates in the regulation of body development, virus defense, hematopoietic processes, organ formation, lipid metabolism, and cell proliferation and apoptosis. Chen et al.[8] found that the miR-195 expression level in glioma tissue was significantly higher than levels in normal brain tissue, and upregulating miR-195 could significantly inhibit the proliferation of glioma cells. Zhang et al.[9] found that miR-24 can promote the growth and metastasis of glioma cells by down-regulating ST7L. Li et al.[10] found that miR-136 can enhance the anti-apoptotic effect of glioma cells on chemotherapy.

Previous studies have shown that miR-129 plays an important anti-tumor effect in many tumors such as medulloblastoma, gastric cancer, colon cancer, endometrial cancer, and lung cancer. miR-129 can inhibit the proliferation of tumor cells and promote their apoptosis by regulating the expression of a variety of cell cycle-related genes[11]. In this experiment, the cell proliferation of the three groups increased over time. The cell proliferation of the miR-129 overexpression group was significantly lower than that of the control group at the 3, 5, and 7-day time points, and the cell proliferation of the miR-129 inhibition group was significantly higher than that of the control group. The apoptosis rate of the miR-129 overexpression group was significantly higher than that of the control group, and the apoptosis rate of the miR-129 inhibition group was significantly lower than that of the control group. Evidence suggests that overexpression of miR-129 can inhibit the proliferation of glioma cells and induce apoptosis, which agrees with the results of Diao et al.[12]. Autophagy is an evolutionarily conserved pathway that relies on lysosomes to reduce the price of damaged organelles wrapped by it, and it plays an important role in the body's physiological and pathological processes. At present, the role of autophagy in tumorigenesis and development is not completely clear. The formation of autophagosomes is a key step in the process of autophagy, in which the LC3 family of ubiquitin-like proteins plays an important role. LC3 is the homolog of yeast ATG in mammals and is currently the most reliable autophagy marker protein. The LC3-II/LC3-I protein ratio is a reliable indicator reflecting the level of autophagy[13].

P62 is a substrate for autophagy degradation and belongs to the family of ubiquitin-like binding proteins. When P62 is coupled with LC3-, they work together to autophagosomes and are degraded in the middle and late stages of autophagy[14]. In this experiment, the expression levels of LC3-II/LC3-I protein and mRNA in the miR-129 overexpression group were significantly higher than those in the control group. The expression levels of p62 protein and mRNA were significantly lower than that of the control group. The expression levels of LC3-II/LC3-I protein and mRNA in the miR-129 group were significantly higher than those in the control group. The expression levels of p62 protein and mRNA were significantly lower than that of the control group. This suggests that overexpression of miR-129 can promote autophagy by regulating the expression of autophagy-related proteins, to delay the proliferation and metastasis of glioma cells. Notch-1 plays an important role in the development of the nervous system and the growth of gliomas. Recent studies have found that Notch-1 plays an important role in alcohol-induced autophagy in cardiomyocytes[15]. In addition, Notch-1 plays an important role in the autophagic death of gliomas. Inhibiting the expression of Notch-1 can effectively inhibit the growth of gliomas. In this experiment, the expression levels of Notch-1 protein and mRNA in the miR-129 overexpression group were significantly lower than those in the control group, and the expression levels of Notch-1 protein and mRNA in the miR-129 inhibition group were significantly higher than those in the control group. This suggests that the regulation effect of miR-129 on the proliferation, apoptosis, and autophagy of glioma cells may be related to the regulation of Notch-1 by miR-129.

In conclusion, miR-129 can inhibit the proliferation of glioma cells and increase the apoptosis rate of cells, as well as promote autophagy by regulating the expression of autophagy-related proteins. The mechanism may be related to the regulation of Notch-1 by miR-129.

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