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Induction effect of MicroRNA-449a on glioma cell proliferation and inhibition on glioma cell apoptosis by promoting PKCα

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Abstract. – OBJECTIVE: This study aimed to investigate the expression of microRNA-449a in brain tissue and plasma of patients with glioma and its mechanism of action on glioma cell proliferation and apoptosis.

PATIENTS AND METHODS: 30 cases of gliomas patients were recruited in the study, 12 cases in which with brain tissues excision due to decompression or exposure during the brain surgery were selected as the control group. RT-PCR was used to detect the microRNA-449a expression in brain tissue and peripheral blood of the two groups. Cell proliferation and apoptosis level were further determined after high or low expression of microRNA-145 in human glioma cell line U-251. RT-PCR and Western blotting were used to detect PKCαmRNA and protein level in U-251.

RESULTS: The expression of microRNA-449a in brain tissue or peripheral blood of patients with brain glioma was significantly lower than that of normal people, and the differences were statistically significant ($p < 0.05$). Upon interfering with microRNA-449a, the glioma cell proliferation was significantly increased while apoptosis was significantly reduced, the PKCα protein levels were increased significantly and the differences were statistically significant (all $p < 0.05$); after overexpression of microRNA-449a, the glioma cell proliferation was significantly decreased while the cell apoptosis was significantly increased, the PKCα protein levels were decreased significantly and the differences were statistically significant (all $p < 0.05$).

CONCLUSIONS: The expression of microRNA-449a is low in patients with glioma, which may inhibit the proliferation of glioma and promote its cell apoptosis via affecting the expression of PKCα.

Key Words:
microRNA-449a, Neuroglioma, Proliferation, PKCα.

Introduction

Neuroglioma (glioma), also known as gliocytoma, is the most common primary central nervous system tumor, accounting for about 50% of the primary brain tumors[1,2]. Etiology of the disease is still unknown at the present, which may be related to tumor origin, genetic factors, biochemical environment, ionizing radiation, nitroso compounds, air pollution, bad living habits and infection, etc[3]. Although the surgical resection is considered to be the preferred treatment for gliomas, it does not significantly improve the survival period of gliomas patients. Therefore, in recent years, the molecular mechanism of this fatal tumor had attracted extensive attention in the research field. Researchers expect to find a more effective treatment[4].

Proteinkinase C (PKC) plays an extremely important role in the cell signaling transduction. A large number of studies have found that abnormal expression of PKC was one of the important reasons for the tumor formation[5-7]. microRNAs (miRNA) is a newly discovered noncoding single small RNA containning 22 nuclear acids. With the in-depth study on miRNA, its abnormal expression has been related to the tumor occurrence and development[8-10]. microRNA-449a is a group of new miRNAs found in recent years. A study has shown the low expression of microRNA-449a in lung cancer patients and it is involved in the growth of lung cancer cells via regulating the expression of HDAC1 molecules[11]. In addition, low expression microRNA-449a was also found in the tissue of patients with bladder cancer and bladder cancer, suggesting microRNA-449a might be an important tumor suppressor[12]. However, up to date, there are few reports about expression of microRNA-449a in glioma patients and its potential function and mechanism of action on glioma cells.

This study investigated the microRNA-449a expression in the cerebrospinal fluid and plasma of patients with glioma and determined its role in the regulation of glioma cell proliferation and apoptosis via influencing the PKC molecules.

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Patients and Methods

Patients

All the tissues were from the surgically excised specimens of glioma patients who were admitted in the neurosurgery department of our hospital between April 2012 and January 2014. There were a total of 30 cases in the study, where 18 cases were male and 12 cases were female, aging from 23 to 65 years. According to the WHO classification, 20 cases were astrocytoma (10 cases were fibrillary astrocytoma, 4 cases were protoplasmic astrocytoma and 6 cases were anaplastic astrocytoma) and 10 cases were glioblastoma. There were 12 cases in the control group, whose brain tissue was excised during brain surgery due to decompression or exposure. Pathologic examination confirmed that the brain tissue was normal with 6 males and 4 females, aging from 30 to 68 years.

Reagents

RNA extraction kit (TRIzol) was purchased from Gibco (Big Cabin, OK, USA); the reverse transcription kits were bought from Thermo Scientific Fermentas Molecular Biology Solutions (ThermoFisher Scientific, Waltham, MA, USA); DNA Marker, PCR kit and DEPC water were bought from Sunshine Biochemical Technology Co., LTD (Nanjing, China). microRNA-449a mimics, inhibitors and U6 internal reference were designed and synthesized by Rib Biochemical Technology Co., LTD (Guangzhou, China).

Total RNA Extraction and RT-PCR

5 ml of peripheral venous blood was taken from fasting patients and normal subjects early in the morning. The blood specimen was added with EDTA anticoagulant, and centrifuged (4000r) at 4°C for 5 min. Upon centrifugation, the superstratum plasma was taken and preserved at -80°C for later use. Brain specimens were immediately added into liquid nitrogen, and stored in refrigerator of -80°C once excised in surgery.

About 150 mg tissue or 200 ml plasma was taken and added with 1 mL Trizol for homogenate. Total RNA was extracted and dissolved in 30 ml of diethylpyrocarbonate (DEPC) water. Ultraviolet spectrophotometer was used for quantitatively and purity detection on extracted Total RNA. Femantes retrovirus kit was then used for reverse transcription and the ultimate product of the cDNA was saved at -20°C. PCR reaction conditions: degeneration at 95°C for 20s, then 60°C for 20 s and 70°C for 1 s, for a total of 35 cycles. Real-Time PCR instrument (Applied Biosystems Company, 7900, Foster City, CA, USA) was used for PCR. The results were given relative quantitative analysis by $2^{-\Delta\Delta Ct13}$. miR-449a primer: 5'TGGCGGTGGCAGTGTATTGTTA3' and 5'GTGCAGGGTCCGAGGT3'; U6 internal references: 5'-CTCGCTTCGGCAGCA CA-3' and 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Transfection

Liposome 2000 (Invitrogen Company, Carlsbad, CA, USA) was used for transfection of microRNA-449a in human glioma cell line U-251, and the interference efficiency was further determined after 48 hours.

Cell Proliferation Detected by CCK-8 Method

As to the human glioma cells transfected by microRNA-449a, necessary serum-free culture medium Dulbecco's Modified Eagle Medium (DMEM) and CCK-8 (Cell Signaling Company, Danvers, MA, USA) were added by 1:10 at 0, 24, 48 and 72 h after transfection. Spectrophotometer was used to measure the OD value at 450 nm and the cell proliferation was determined according to the cell growth curve.

Western Blot Analysis

Human glioma cells were collected after transfection, and added with 1×SDS lysis solution. Then SDS-PAGE electrophoresis was performed. The proteins were transferred onto membrane under voltage of 110V for 90 min, and then the membrane was blocked at 37°C for 90 min. Rabbit-anti-human PKCα and Bcl-2 (Biolegend, San Diego, CA, USA) were added at 4°C for overnight incubation. HRP-labeled mice-anti-rabbit secondary antibody (Nanjing SunShine Biological) (dilution at 1:2000) was then added and incubated at 37°C for 40 min. electrochemiluminescence (ECL) was used for detection. Anti-human b-actin (Sigma, St. Louis, MO, USA, dilution at 1:1500) was used as internal standard protein.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Student's *t*-test was used for comparison between groups. $p < 0.05$ was considered to have statistically significant differences.

Results

Expression of microRNA-449a mRNA in Brain Tissues of Patients With Glioma

Compared to normal brain tissue, microRNA-449a was significantly decreased in brain tissue of patients with glioma and the differences were of statistical significance ($t = 10.87$, $p < 0.05$), which suggested that lower expression of microRNA-449a might play a significant inhibitory role in the development of glioma (Table I).

Expression of Plasma microRNA-449a mRNA in Patients With Glioma

Expression of plasma microRNA-449a mRNA in patients with glioma was further detected. The results showed that plasma microRNA-449a mRNA level was significantly lower in patients with glioma than that of normal people, and the differences were of statistical significance ($t = 9.07$, $p < 0.05$), which indicated that lower expression of microRNA-449a might be closely related to the development of glioma.

Effects of Low Expression of microRNA-449a on Proliferation and Apoptosis of Glioma Cells

After transfection with microRNA-449a inhibitor, microRNA-449a was significantly reduced in glioma cells U-251 (Figure 1A), suggesting successful interfering effect. After interfering with microRNA-449a in glioma cells, the cell proliferation was significantly increased (Figure 1B) while the apoptosis was significantly reduced (Figure 1C), and PKCα level was significantly increased (Figure 1D), and the differences were statistically significant (all $p < 0.05$). It suggested that microRNA-449a inhibited the proliferation of glioma cells and promoted its cell apoptosis via inhibiting PKCα expression.

Table I. Expression of microRNA-449a mRNA in brain tissues of patients with glioma.

Groups	microRNA-449a mRNA	t/p
Normal brain tissues	1.16 ± 0.21	
Glioma	0.38 ± 0.21*	10.87/0.000

Note: *Compared to normal brain tissues, $t = 10.87$, $p < 0.05$.

Table II. Expression of plasma microRNA-449a mRNA in patients with glioma.

Groups	microRNA-449a mRNA	t/p
Normal brain tissues	1.25 ± 0.32	
Glioma	0.41 ± 0.25*	9.07/0.000

Note: *Compared to hepatic cirrhosis resulting from hepatitis B, $t = 9.07$, $p < 0.05$.

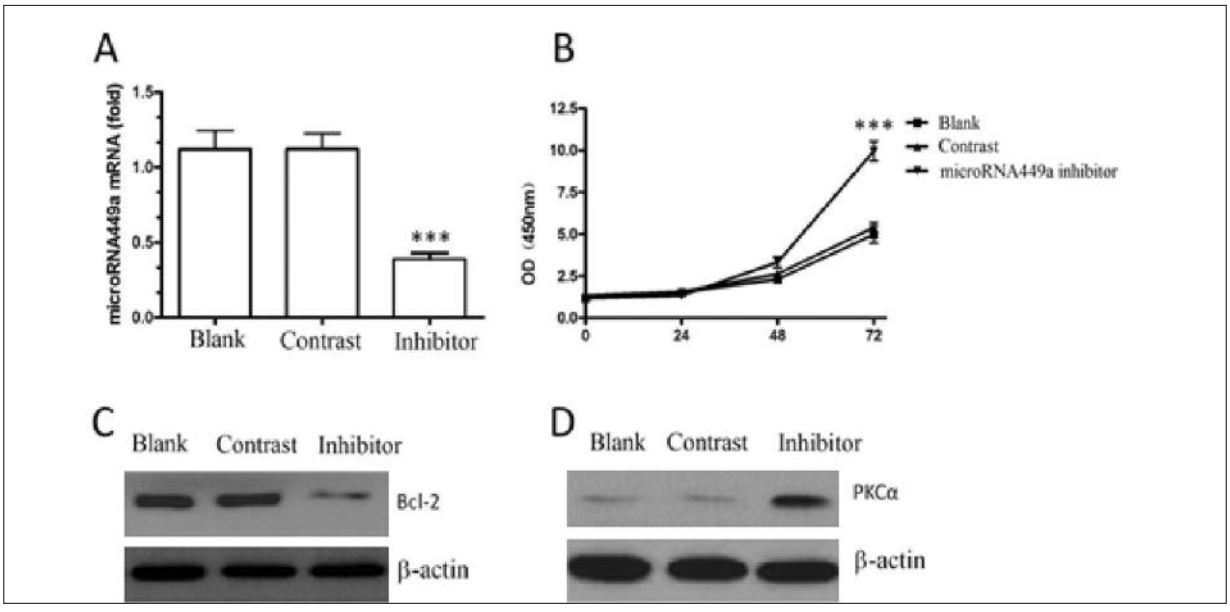

Figure 1. Low expression of microRNA-449a on proliferation and apoptosis of glioma cells. ***Compared to the control group, $p < 0.000$.

Effects of High Expression of microRNA-449a on Proliferation and Apoptosis of Glioma Cells

After transfection with microRNA-449a mimics, microRNA-449a was significantly increased in glioma cells U-251 as expected (Figure 2A). After overexpression, glioma cell proliferation was significantly reduced (Figure 2B) while the apoptosis was significantly increased (Figure 2C), and PKCα level was significantly decreased (Figure 2D), and the differences were statistically significant (all $p < 0.05$). It indicated that microRNA-449a inhibited the proliferation of glioma cells via inhibiting PKCα expression and affected its apoptosis via promoting Bcl-2.

Discussion

Glioma, accounting for 40-50% cerebral tumors, is a common malignant tumor. Its diagnosis and treatment has always been one of the challenging clinical problems. According to the pathological classification, it is comprised of astrocytoma, medulloblastoma, glioblastoma multiforme, ependymoma. Usually, there are not any typical symptoms at the early stage of glioma. As the disease advances, an increased intracranial pressure and other general symptoms, such as headache, vomiting, decreased visual acuity, diplopia, seizures, and mental symptoms, will start to show. Glioma often shows invasive growth and there is no boundary between it and normal brain tissue. Therefore, it is difficult to be completely removed. The molecular pathogenesis mechanism of glioma has attracted considerable attention in clinical research in order to find a more effective method for treatment at molecular level.

MicroRNA is a small molecular RNA with 22 nucleotides. It is widely distributed in tissues, plasma or serum and other body fluids. MicroRNA can regulate the expression of human target genes via inhibiting mRNA translation and inducing mRNA degradation. Although the biological functions of miRNA are not fully understood, miRNA is expressed normally in most of the human tumors; therefore, miRNA is considered to play an important role in the occurrence and development of cancer[14-19]. The present study suggests that miRNA may play a role of carcinogenicity via inhibiting tumor suppressor genes or proto-oncogenes.

This study explored the function and significance of microRNA-449a in patients with glioma by detecting the expression of microRNA-449a in brain tissues and plasma of patients with glioma. The results suggested that expression of microRNA-449a was lower in brain tissue and plasma of patients with glioma than that of normal people. We, therefore, speculated that lower expression of microRNA-449a in patients with

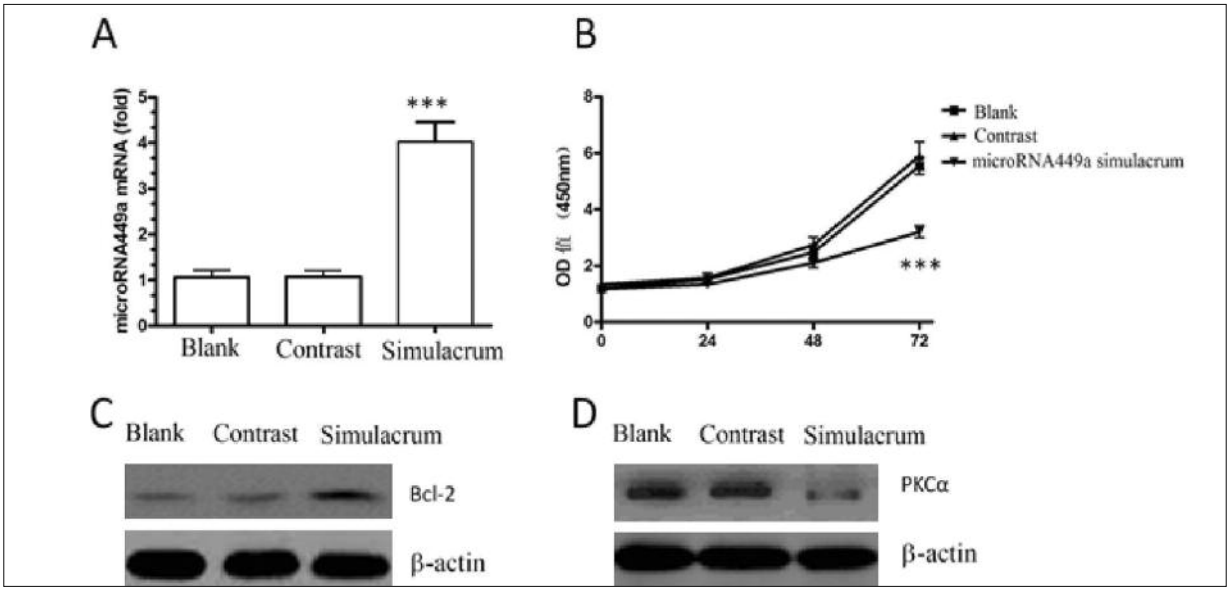

Figure 2. High expression of microRNA-449a on proliferation and apoptosis of glioma cells. ***Compared to the control group, $p < 0.000$.

glioma might be closely related to occurrence and development of the gliomas. Chen et al[12] found that microRNA-449a was significantly lower in bladder cancer tissues than normal group, suggesting it might have tumor inhibition effects, which is consistent with the results of our research.

In addition, this research also explored the effects of microRNA-449a on proliferation and apoptosis of glioma cells via over-expressing or inhibiting microRNA-449a in glioma cells. The results showed that microRNA-449a could inhibit the proliferation of glioma cells and affect its apoptosis via promoting expression of Bcl-2. Abnormal expression of protein kinase C (PKC) in tumors is closely related to proliferation of tumor cells[20]. We further explored relevant mechanism of microRNA-449a affecting glioma cell proliferation and apoptosis. Through detection on PKCα expression of glioma cells over-expressing or inhibiting microRNA-449a, the results showed that glioma cells with interfered microRNA-449a expression had high PKCa protein expression, while glioma cells with high expression of microRNA-449a had lower PKCa protein expression, thus, confirming that microRNA-449a could inhibit proliferation of glioma cells via inhibiting PKCα expression, and promote its apoptosis by affecting Bcl-2.

Conclusions

microRNA-449a might act as tumor suppressor in glioma. It is mainly involved in glioma cell proliferation and apoptosis by inhibiting PKCα level. Clinically, the tissue and plasma microRNA-449a level in glioma patients could be used as the indicator for treatment and prognosis of glioma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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