

Original Article

Function and mechanism of miR-23a-3p in the brain gliomas

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Abstract: Objective: To investigate the expression of miR-23a-3p in human glioma tissues and its effects on the growth of glioma as well as the proliferation, migration, cell cycle and apoptosis of U251 cells. Methods: The intracellular gene expression level was detected by qPCR. CCK8 was used to detect the cell proliferation. Transwell assay was adopted to detect cell migration. Flow cytometry was used to detect the cell cycle and apoptosis. And the targeted regulation of miR-23a-3p to the CTNNBIP1 was detected by dual-luciferase test. Results: In glioma, miR-23a-3p expression level was proportional to the malignant degree of glioma and the expression level in HGG (high grade glioma) was significantly higher than that of LGG (lower grade glioma); correspondingly, the expression of CTNNBIP1 was inversely proportional to the malignant glioma degree and the expression level in HGG was lower than that of LGG. Analyzing the correlation between miR-23a-3p and CTNNBIP1, we found there was negative correlation between the two. The dual-luciferase experiment confirmed that the two were targeting, and miR-23a-3p could target regulate the expression of CTNNBIP1. The lentivirus transfection technique was used to interfere the expression of miR-23a-3p in the cells and analyze the function of miR-23a-3p in the U251 cells. It was found that the down regulation of miR-23a-3p expression suppressed the proliferation, migration, cell cycle, and promoted apoptosis of U251 cells. On the basis of the interference of miR-23a-3p, we interfered the expression of CTNNBIP1, then find that compared with interference of miR-23a-3p group, the cells inhibition of proliferation, migration and cycle had been weakened; however, the effect on promoting apoptosis had been inhibited. The nude mice in vivo experiment confirmed that the low expression of CTNNBIP1 can reduce the expression of miR-23a-3p and the inhibitory effect on tumor growth worked. Conclusion: miR-23a-3p is defined as oncogene in glioma, which can promote the development of glioma cells. And this promotion effect is achieved by targeting CTNNBIP WNT pathway to inhibit synthesis of protein.

Keywords: MicroRNAs (miRNAs), miR-23a-3p, glioma

Introduction

Glioma is one of the important harmful factors to human health. With proliferation and infiltration of intracranial and high degree of malignancy, it is easy to relapse. Now, owing to the poor therapeutic effect of conventional treatment and prognosis in glioma, the clinical practitioners and researchers are facing great challenges. Understanding the mechanism of the occurrence and development of glioma is helpful for the treatment of glioma. There are a series of gene level changes accompanied with the occurrence and development of glioma, such as some oncogene expression changes: TP53, s-myc, p21, p27, miRNA: miR-34a, mir-

181d, let-7, etc [1, 2] or abnormal expression of signaling pathways: SOX2-lin28/Let 7 signaling pathway, WNT signaling pathway and so on [3]. The WNT pathway plays an important role in the occurrence and development of gliomas. The activation of the WNT signaling pathway is a major cause of [4] primary neuroectodermal tumor of the brain. β -catenin protein- (CTNNB1) is a vital regulatory protein in the WNT pathway. β -catenin Interacting Protein 1 (CTNNBIP1) can combine with CTNNB1, stop the function between the CTNNB1 protein and TCF family, negatively regulate the WNT pathway and thus the development of glioma is inhibited [5]. In recent years, the research of the miRNA and the occurrence and development of molecular biology

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mechanism of glioma has provided a new biological therapeutic target for glioma [6]. It was reported that the expression of MIR-23a-3p in cerebro-spinal fluid (CSF) of patients with fibromyalgia was lower than that of normal people [7]. The over expression of MiR-23a-3p in the aging of fibrocyte and targeted regulation of HAS2 (synthase hyaluronan 2) are related with cell aging [8]. Studies have indicated that MiR-23a-3p inhibited the expression of E-cadherin in the development of prostate cancer, and epithelial-mesenchymal transition (EMT) of cells [9]. However, there is little report about miR-23a-3p in glioma and its function in the development of glioma remains unclear. Q-PCR was used to detect the expression of MiR-23a-3p in glioma tissues, transfection technique (in vitro) was adopted and model of hypodermatic xenografts of human brain glioma in nude mice was established, observing effects and related mechanism of MiR-23a-3p on the proliferation, migration, cell cycle and apoptosis of U251 cells to explore the possibility of MiR-23a-3p as the gene therapy target for glioma.

Materials and methods

Experimental specimens

100 cases of brain glioma specimen and 50 control specimen (Shanghai RuiSai Biotechnology Co., Ltd), pathological grading: grade I 22 cases, grade II 27 cases, grade III 26 cases, grade IV 25 cases; lower-grade glioma (LGG) 49 cases, and high-grade glioma (HGG) 51 cases.

Cell Strain and main reagents

Human glioma cells U251 cell line (American Type Culture Collection, ATCC), was frozen in the laboratory. Transwell chamber (Corning Costar Company); TaqMan microRNA reverse transcription kit and test kit (Applied Biosystems Company); Annexin V FITC/PI (BD Biosciences, USA); RNase A (Boehringer Mannheim Company USA); MTT and PI (Sigma Company, USA); lentivirus mediated MiR-23a-3p interference vector was commissioned Shanghai Zimmer to synthesize.

Using qPCR to detect the expression of CTNNBIP1 as well as MiR-23a-3p in glioma tissues and U251 cells

Total miRNA of glioma tissues and U251 cells were extracted according to the instruction of

one-step method of miRNA manual. TaqMan microRNA reverse transcription kit were used to reverse transcript the total miRNA into cDNA and added MiR-23a-3p or internal reference U6 primer to amplify. Reaction conditions: predegeneration at 95°C for 10 min, denaturation at 95°C for 15 s and annealing/extension 60°C for 1 min, with a total of 40 cycles.

Trizol reagent was used to extract the total RNA of glioma tissues and U251 cells. cDNA was synthesized by cDNA synthetic reagent kit. β -actin was regarded as the internal reference. The corresponding primers were designed according to the sequence of Gene, hCTNNBIP1-F1: CCGTTAGGCGGGATTATG, hCTNNBIP1-R1: TGGGGCTTTTATG TGGG TT; Reaction system: forward and reverse primers 0.4 μ L respectively, SYBR Green Realtime PCR Master Mix 10 μ L, cDNA 2 μ L, ddH₂O 7.2 μ L. Reaction conditions: 95°C for 10 min, 95°C for 15 s, 58.5°C for 15 s, 72°C for 45 s, 2°C for 10 min, with a total of 40 cycles.

After PCR, analyze gene amplification conditions in ABI 7300 System software, get the corresponding Ct value, take U6 or β -actin as an internal reference to correct the copy number of PCR template, and the relative expression of genes should be calculated by $2^{-\Delta\Delta Ct}$ methods.

Cell culture and experimental grouping

U251 cells cultured in DMEM medium containing 10% fetal bovine serum, and was put in an incubator with the condition of 37°C and 5% CO₂, and the exponential phase cell was selected to carry out the experiment that was divided into three groups: lentivirus NC infection group (negative control group), transfecting empty lentivirus vector into U251 cells; miR-23a-3p interference group: transfecting lentivirus vector containing miR-23a-3p interference sequence into U251 cells. miR-23a-3p interference + CTNNBIP1 interference group: the same treatment as the miR-23a-3p interference group, but added the CTNNBIP1 inhibitor (KIAA0495) in the U251 cell culture solution. Collect the cells at different time points in each group. Repeat the experiment 3 times in each group.

Using CCK-8 to detect the proliferation of U251 cell

U251 cells in each group were inoculated in the 96-well plate with the density of 5×10^4 /ml,

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added in the culture solution. After treatment, put it in an incubator with the condition of 37°C and 5% CO₂ cultured for 1 d, 2 d, 3 d, 4 d and 5 d respectively. Having been added CCK-8 10 L in each hole, cells were cultured in the incubator and continuing to be incubate for 4 hours. Use the microplat reader t to measure the absorbance of each hole in 450 nm, and then take the average value of the 5 holes.

Transwell experiment

Apply 24-hole Transwell chamber to carry out the U251 cell migration experiment. In the upper chamber on each hole, there was 200 µL serum-free DMEM culture medium with 5*10⁴ cells, and in the lower chamber there was 200 µL DMEM medium containing 10% fetal bovine serum. After 24 hours of culture, wipe the non-invasive cells of upper chamber with wet cotton swab. After fixation, stain with 0.1% crystal violet, photograph under the microscope, dehydrate with 33% acetic acid, to elute crystal violet completely, and then measure the OD value with the eluent at 570 nm by the microplat reader.

Flow cytometry to detect the cell cycle of U251 cells

U251 cells in each group were inoculated in the 6 -well plate with the density of 5*10⁵/ml. After the corresponding treatment for 48 h, collected which was washed by PBS in the flow tube. After the 75% alcohol suspending again, placed it at -20°C for 1 h, use the PBS to wash again. U251 cell cycle was detected by flow cytometer after the suspending again of 50 µg/ml Plde HBSS solution.

Flow cytometry to detect the apoptosis of U251 cells

U251 cells in each group were inoculated in the 6 -well plate with the density of 5*10⁵/ml. After the corresponding treatment for 48 h, collect which was washed by PBS in the flow tube. After double stained by V FITC/PI Annexin, the apoptosis of U251 cells was detected by flow cytometer.

Construction and activity detection of luciferase reporter vector

According to the CTNNBIP1 sequence and the predict outcomes of Target Scan software, the

3'UTR sequence of constructed DNA sequences was amplified by PCR, cloning to pGL-Basic reporter gene plasmid (pGL-UTR), sequencing verification, constructing luciferase reporter gene vector pGL3-WT- CTNNBIP1-3'UTR- wild type and pGL3-MUT- CTNNBIP1-3'UTR- mutant, and entrust the Dalian Treasure Biological Company to complete the sequencing work. Lipo2000 liposome respectively mediated these two plasmids and chemosynthetic miR-23a-3p simulacrum, sequence with nonsense to transfecte U251 cells of 70% confluence. After 48 h, apply dual luciferase assay kit of Promega Company to detect the luciferin activity.

Observing and establishing model of hypodermatic xenografts of human brain glioma in nude mice

24 BALB/C-nu/nu mice were (Shanghai Slack Experiment Co. Ltd.) 4-6-week-old male and female mice with an average body weight of 18-21 g. They were randomly divided into three groups. The groups were inoculated U251 cells with low expression of miR-23a-3p, low expression of miR-23a-3p + interference CTNNBIP1 and empty lentivirus vector infection respectively. Mice were kept under SPF level conditions.

Parallely cultured a large number of U251 cells with low expression of miR-23a-3p, low expression of miR-23a-3p + interference CTNNBIP1 and empty lentivirus vector infection. When it came to the exponential phase, regularly digested cells, centrifugated and counted, use serum-free DMEM medium to wash the cells once and then make into cell suspension, with a concentration of 5*10⁶ cells/100 µL. Use 75% alcohol to disinfect injection site on the right side of the ribs of nude mice and injected 100 µL subcutaneously in each one. After inoculation of U251 cells, tumor growth in nude mice was observed every day on a regular basis with a vernier caliper measuring the long as well as short diameters of tumors, with every 4 days per time, and then the volume of tumors was calculated.

Statistical analysis

SPSS19.0 software was used for statistical analysis; measurement data were expressed as mean ± standard deviations. T-test was

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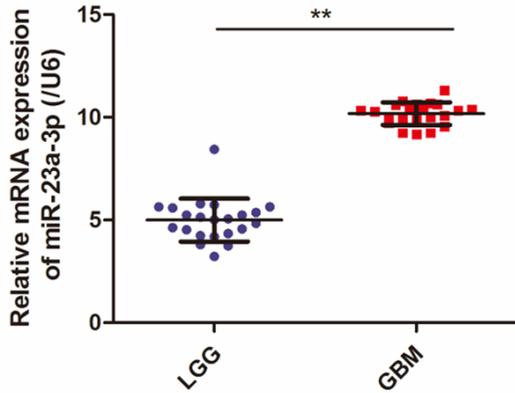


Figure 1. Comparison of the expression levels of miR-23a-3p in different pathological grades of gliomas (** $P < 0.05$).

used to detect the comparison of mean differences between two groups; comparison of mean differences among multi-groups was examined by One-way ANOVA. Count data was expressed by percentage, and the comparison between groups was examined by chi-square test; the correlation between the two was detected by Pearson correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Expression of miR-23a-3p in glioma tissues

For detecting the expression of miR-23a-3p in glioma tissue specimens, qPCR were examined by it, and the results showed as follows: divided the clinical and pathological grade I-II grades into LGG (Lower grade glioma); divided the clinical and pathological grade III-IV grades into HGG (High grade glioma). In different pathological grades of gliomas, miR-23a-3p expression would rise with increasing pathological grade ($P < 0.05$), as shown in **Figure 1**.

Influence of low expression of miR-23a-3p on proliferation, migration, cycle and apoptosis of U251 cell

In order to study that whether the expression of miR-23a-3p would down regulate after lentivirus-mediated interference of miR-23a-3p in U251 cells, expression of miR-23a-3p were detected by qPCR after 48-hour transfection in two groups. The results showed as follows: it can significantly decrease the expression of miR-23a-3p in U251 cells after lentivirus-mediated

interference of miR-23a-3p, as shown in **Figure 2A**; compared with the control group, it can significantly inhibited cell proliferation, migration and cell cycle, and promoted apoptosis in low expression of miR-23a-3p (**Figure 2**).

miR-23a-3p targeting CTNNBIP1 3'-UTR

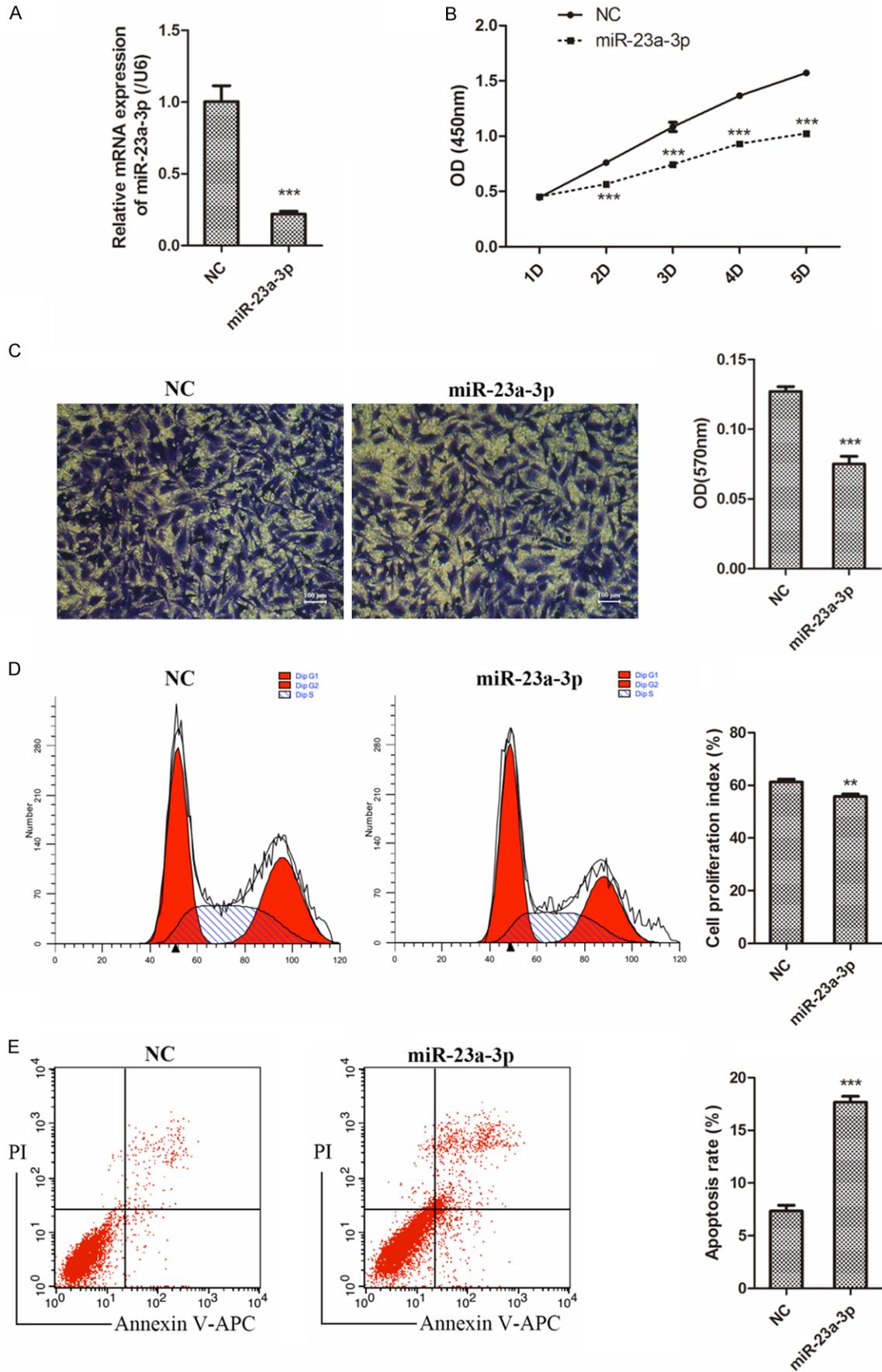
In order to further study the mechanism of miR-23a-3p regulating the biological behavior of glioma cells, Target Scan software was used to predict relations of targeted regulatory between miR-23a-3p and CTNNBIP1. Q-PCR and luciferase reporter assay were used to verify that miR-23a-3p can targeted regulate the expression of CTNNBIP1. Q-PCR results showed that the down regulation of miR-23a-3p expression in U251 cells promoting the expression of CTNNBIP1 in cells. What was more, in the tissue samples of different pathological grades, compared with the low grade glioma, the expression of CTNNBIP1 with high grade reduced, and the correlation analysis of the expression of CTNNBIP1 and miR-23a-3p in the high grade glioma showed negative correlation, as shown in **Figure 3**.

To verify whether miR-23a-3p was worked by CTNNBIP1, the expression of CTNNBIP1 was inhibited on the basis of interfering miR-23a-3p. Cell experimental results showed that the effects on the inhibitory of proliferation, cell cycle and migration and promotion of apoptosis were weakened for U251 cells, as shown in **Figure 4**.

Observation and measurement of the growth of hypodermatic xenografts of human brain glioma in nude mice

To verify the effect of miR-23a-3p on the growth of glioma cells by CTNNBIP1, NC lentivirus infected cells was taken as control. The other two groups of nude mice were injected with 5×10^6 U251 cells with stable low expression of miR-23a-3p, and U251 cells with low expression of miR-23a-3p + interference CTNNBIP1 respectively. At 14th day after injection, the size of the tumor started to measure. 40 days later, the solid tumor of the glioma was taken out and the results showed that, compared with the control group, the tumor volume in the group of U251 cells with stable low expression of miR-23a-3p was significantly decreased. However, on the basis of interfering CTNNBIP1 expres-

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Figure 2. Influence of interference expression of MiR-23a-3p on the proliferation, migration, cell cycle and apoptosis of U251 cells (**P < 0.05, ***P < 0.01).

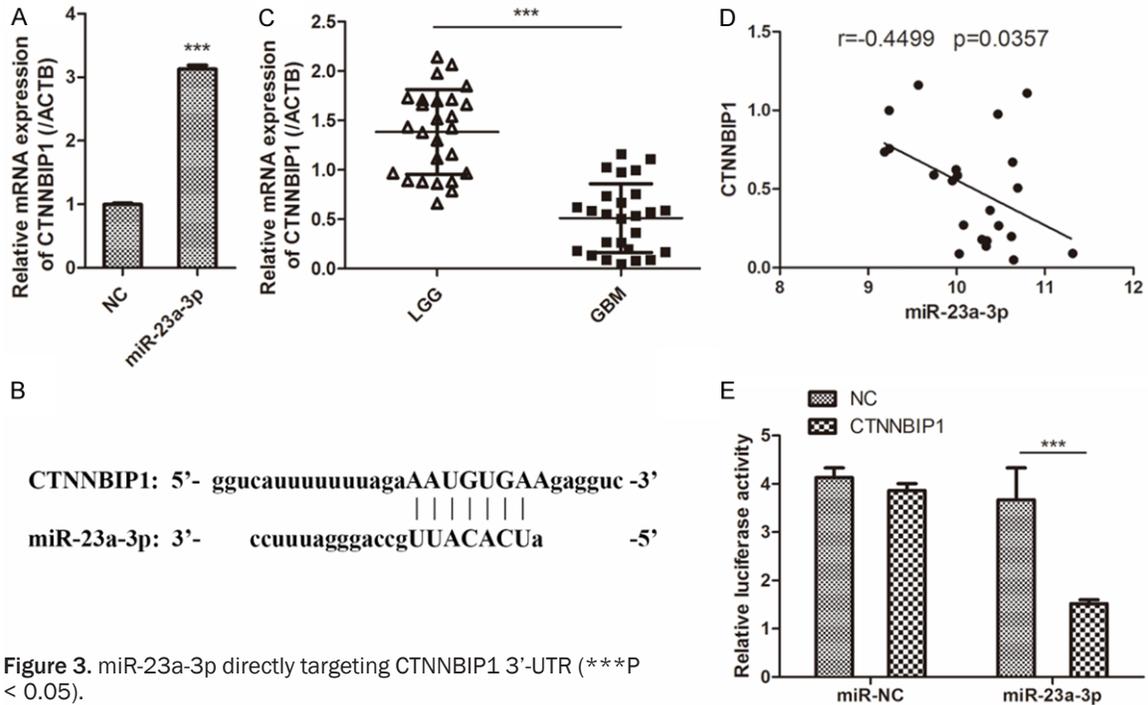


Figure 3. miR-23a-3p directly targeting CTNNBIP1 3'-UTR (***)P < 0.05).

sion, the effect on miR-23a-3p of growth of tumor volume was inhibited (P < 0.05), as shown in **Figure 5**.

Discussion

Human glioma is the most common primary malignant tumor of the central nervous system, accounting for about 40% of the intracranial primary malignant tumor. The prognosis of brain gliomas is poor, and the average survival time of patients is about 1 year after comprehensive therapy like surgery, radiotherapy and chemotherapy [10, 11]. With the characteristics of high malignant degree, invasive growth and high recurrence rate of human gliomas, there is a great challenge to the treatment of patients. It is reported that treatment failure of patients with glioma is often due to the diffuse invasive growth and intracranial recurrence of tumor [12, 13]. Therefore, how to inhibit the biological behavior of glioma cells is one of the important factors in the treatment of glioma.

As a kind of endogenous non coding of single stranded molecules RNA, MicroRNA is highly conserved in evolution, the action mechanism

of which is combined with the 3'UTR terminal of target mRNA completely or incompletely, suppresses translation or degrades the target mRNA, and thus regulates the life activities [14]. Some studies indicate that half of miRNAs are located in the fragile site of the genome or the region of tumor related genome, indicating that miRNA may play an important role in the occurrence and development of tumors [15, 16]. Recent studies have shown that miRNA is closely related to the biological behavior of proliferation, invasion, cell cycle and apoptosis in tumor cells [17, 18]. Other studies have reported that abnormal expression of miRNA [19] can be detected in most of the malignant tumors. Obviously, the discovery of miRNA provides a new thinking and target in the treatment of glioma. At present, applying lentivirus-mediated interference vector to reduce the expression of specific MiRNA so that the inhibition of tumor growth and metastasis can be achieved, has made progress in the experimental study of animal tumor model [20].

MiR-23a-3p which widely regulates the processes of various physiological and pathological in body, plays an oncogene role in a variety of

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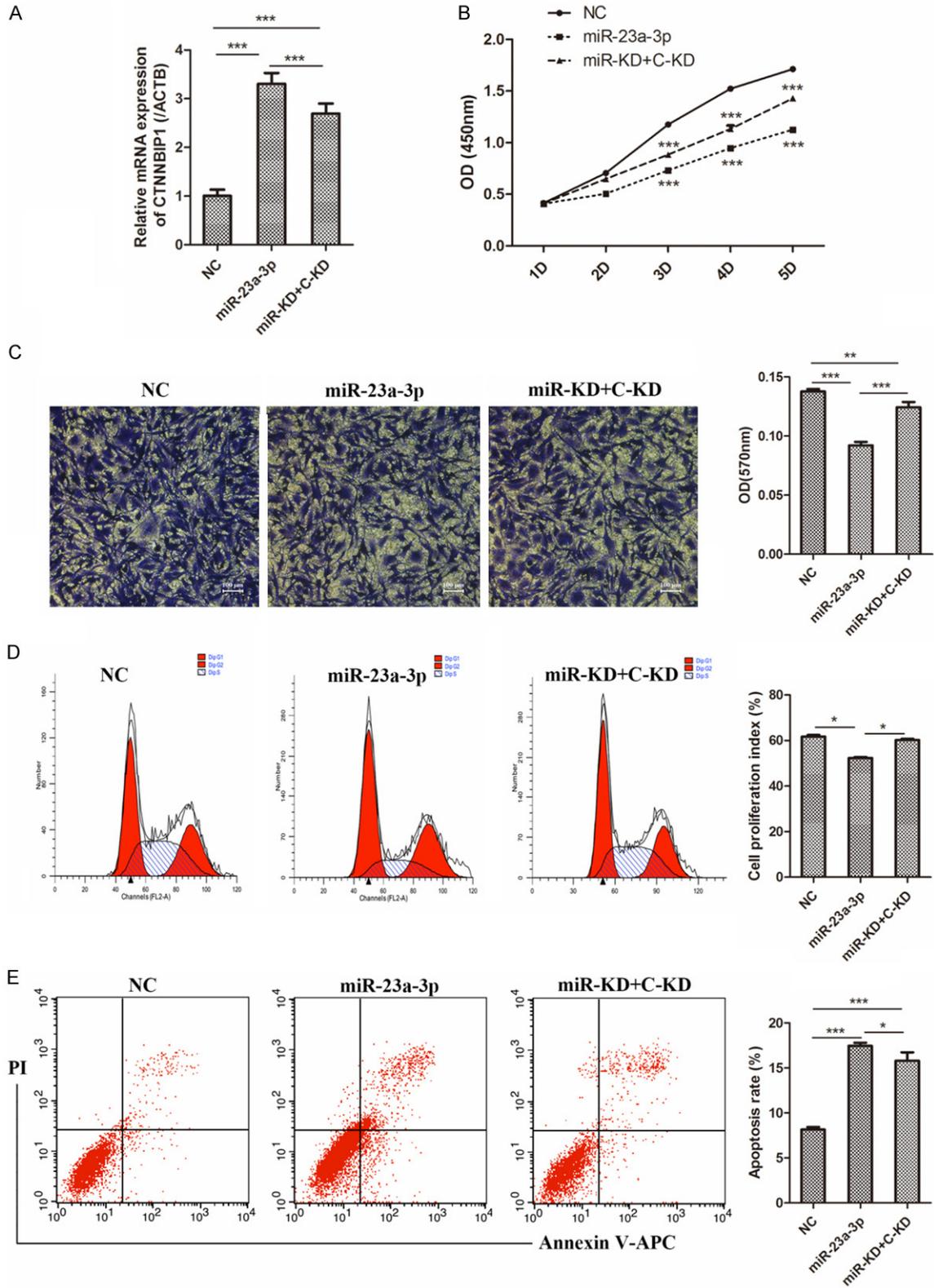


Figure 4. Effects of interfering CTNNBIP1 expression on the proliferation, migration, cell cycle and apoptosis of U251 cells with low expression of miR-23a-3p (* $P < 0.05$, ** $P < 0.05$, *** $P < 0.01$).

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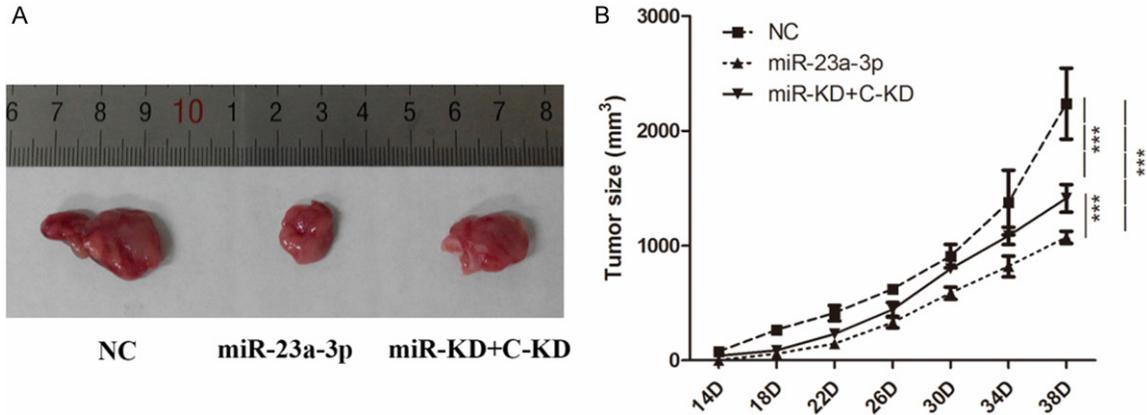


Figure 5. The growth of tumor in nude mice after 14 days inoculated cells (***) $P < 0.05$.

tumors. It can promote the proliferation and migration of tumor cells, and inhibit the apoptosis. However, there is little research about the function of miR-23a-3p in brain glioma. Some studies have showed that as oncogene, miR-23a-3p over expresses in the early stage of Huh cells in liver cancer and speeds up the proliferation of tumor cells [21]. In this experiment, firstly, qPCR was used to detect the expression amount of miR-23a-3p in the brain glioma tissues and the U251 cells. The results showed that the expression level of miR-23a-3p in grade III and IV glioma tissues was significantly higher than that of grade I and II, indicating that miR-23a-3p might become one of the predictors of tumor malignant degree, and confirming that miR-23a-3p might have effects of oncogene in the glioma. Then, the results of lentivirus-mediated interference of miR-23a-3p in U251 cells showed that it could significantly reduce the expression level in U251. Besides, low expression of miR-23a-3p could significantly inhibit the proliferation, migration, cell cycle and apoptosis of U251 cells. Obviously, miR-23a-3p in U251 glioma cells also have a function of oncogene. Then, the results of the animal experiment, cell experiment and dual luciferase experiment showed that miR-23a-3p regulate the biological behavior of U251 Cells by directly targeting CTNNBIP1 protein, hinting that the promoting effect was achieved by targeted regulating CTNNBIP1 and inhibit the synthesis of protein.

As is mentioned above, miR-23a-3p highly expresses in brain gliomas, which may be involved in the process of occurrence and development

of glioma, and down regulation the expression of miR-23a-3p may be a new way to treat glioma.

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Disclosure of conflict of interest

None.

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