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# MiR-448 downregulates CTTN to inhibit cell proliferation and promote apoptosis in glioma

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**Abstract. – OBJECTIVE: miRNAs have been confirmed to be related to cell proliferation and apoptosis. In this study, we detected the potential effect of miR-448 on glioma cell proliferation and apoptosis.**

**MATERIALS AND METHODS: miR-448 and CTTN expression levels were detected in glioma cell lines with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Cells were transfected with miR-448 mimics and inhibitor by using lipofectamine 2000 respectively. The proliferative ability of transfected cells was detected via methyl thiazolyl tetrazolium (MTT) and cell counting kit-8 (CCK8) assays. Cell apoptosis and cell-cycle were tested using flow cytometry. The regulatory correlation between miR-448 and CTTN was explored by bioinformatics analysis and luciferase reporter assay.**

**RESULTS: Lower expression of miR-448 and higher level of CTTN were detected in glioma cells. MiR-448 could regulate cell proliferation, cell apoptosis, and cell cycle. CTTN was negatively regulated by miR-448.**

**CONCLUSIONS: miR-448 downregulates CTTN to inhibit cell proliferation and promote apoptosis in glioma, which indicates a potential therapeutic target of glioma.**

*Key Words:*
MicroRNA, miR-448, Glioma, Proliferation, Apoptosis.

## Introduction

Glioma is the most common tumor of the central nervous system. With higher mortality, the average survival of glioblastoma is only 12-15 months. Meanwhile, the average survival time of metaplastic glioma is only 2-5 years[1]. Although surgical resection combined with radiotherapy and chemotherapy is developed rapidly now, it does not significantly improve the survival of patients with glioma. The limited treatment of glioma drives many explorations of the molecular mechanisms so as to achieve a more reasonable and effective treatment[2].

Current researches have showed that abnormal expression levels of miRNAs are implicated in the development of glioma, which conduces to become new markers and therapeutic targets for diagnosis and prognosis of glioma. This significant view has brought new prospects for the treatment of glioma[3].

Up to date, lots of studies have identified that miRNAs could influence the progression of glioma. miR-128 repressed glioma cells proliferation and invasion through controlling COX-2[4]. In glioblastoma, miR-129 could block PKC/ERK/NF-κB and JNK pathways by influencing Wnt5a[5]. miR-200a was reduced in glioma tissues, and upregulated Gαi1 expression could promote cell proliferation[6]. miR-1294 suppressed cell proliferative ability and promoted the chemosensitivity to temozolomide by regulating TPX2 in glioma[7]. miR-129-3p inhibited cell growth via binding to E2F5 gene in glioblastoma[8]. In glioma, miR-484/MAP2/c-Myc-positive regulatory loop contributed to tumor-initiating properties through controlling ERK1/2 signaling pathway[9]. miR-375 regulated cell proliferation and migration through targeting RWDD3 in glioma[10]. miR-375 could modulate glioma cell proliferation and apoptosis via regulating CTGF-EGFR signaling pathway[11]. miR-509-3p could suppress cell proliferation and invasion in glioma[12]. In breast cancer, miR-448 could repress EMT process via regulating E-cadherin repressor ZEB1/2[13]. MiR-448 could inhibit osteosarcoma cell proliferation and invasion by controlling EPHA7[14]. In colorectal cancer, miR-448 could repress proliferation and invasion by modulating IGF1R[15]. In gastric cancer, miR-448 could repress proliferation and invasion through targeting ADAM10[16]. In this study, we explored the expression of miR-448 in gliomas and identified the effect of miR-448 on proliferation and apoptosis of glioma cells, which

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provided new evidence for understanding the glioma development.

## Materials and Methods

### Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was conducted via using the SYBR Green PCR kit (TaKaRa Biotechnology, Co., Ltd., Dalian, China) and the ABI 7500 FAST Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sequences of the primers used are as follows: miR-448 forward, 5′-TTGCATATGTAGGATGTCCCAT-3′ and reverse, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGGGACA-3′; CTTN forward, 5′-TAATCCAATGAGGAATTTCCAG-3′ and reverse, 5′-TAGAGCCTGGTGCCTGGG-3′; U6 forward: 5′-CGCTTCGGCAGCACATATAC-3′, reverse: 5′-TTCACGAATTTGCGTGTCAT-3′; GAPDH forward, 5′-GGTGAAGGTCGGAGTCAACG-3′, and reverse: 5′-CAAAGTTGTCATGGATGHACC-3′. The conditions were 95°C for 30 sec; 40 cycles of 95°C for 5 sec, and 60°C for 30 sec; 95°C for 15 sec, 60°C for 60 sec, and 95°C for 15 sec. U6 was regarded as the internal reference.

### Cell Transfection

Cell lines (U87, T98, U251, and U373) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in a 37°C 5% $CO_2$ condition. Normal human astrocytes (NHA) were obtained from Cell Bank of Fudan University (Shanghai, China), and then cultured in astrocyte media (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) with 10% FBS (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C 5% $CO_2$ condition. According to the manufacturer instructions, U87 cells were transfected with miR-448 mimics by using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), U373 cells were transfected with miR-448 inhibitor by using Lipofectamine 2000.

### *Methyl Thiazolyl Tetrazolium (MTT) Assay*

The transfected cells were seeded into 96-well plates at a density of 5,000 cells per well. 20 μL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Then, after 4 h, 100 μL dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added to each well at 37°C condition. ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for the detection of the optical density (OD) value of each well.

### Cell Counting Kit-8 (CCK8)

The transfected cells were counted and then inoculated in a 96-well plate (5×103 cells per well), and 3 parallel wells were established. Then, CCK-8 reagent (10 μL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and continued to culture for 2 h. The optical density (OD) value at 24, 48 and 72 h was assessed at 570 nm. The survival curves of cells were obtained. Every experiment was performed three times.

### Cell Cycle Analysis

The transfected cells were washed with phosphate buffered saline (PBS) and then fixed at −20°C using 75% pre-cooled ethanol. After 24 h, cells were stained with propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 37°C. Cell cycle was evaluated for DNA content using a FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Every experiment was performed three times. The results were obtained as the percentage of the cell cycle.

### Cell Apoptosis

After transfection, glioma cells are suspended in the binding solution, and then added 10 μL Annexin V (BD, Franklin Lakes, NJ, USA) at 4°C for 30 min. Then, added 5 μL PI (Sigma-Aldrich, St. Louis, MO, USA), cell apoptosis was detected by using FCM (BD, Franklin Lakes, NJ, USA). Every experiment was repeated at least three times.

### Luciferase Assay

Using Lipofectamine 2000, cells were transfected with miR-448, CTTN 3`-UTR-wild type and mutant plasmids (Biomics Biotechnologies Co., Ltd. Nantong, China), then they were incubated at 37°C 5% $CO_2$ condition. According to the manufacturer's protocol, the activity was detected by using a luciferase assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Renilla luciferase activity was acted as the control.

### Statistical Analysis

The data are expressed using mean ± standard error in Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA) with Chi-square analysis and Student's *t*-test analy-

sis. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Statistical significance is defined as $p<0.05$.

## Results

### Downregulated miR-448 and Overexpressed CTTN Were Detected in Glioma Cells

The expression levels of miR-448 and CTTN were measured in glioma cell lines with qRT-qPCR. The results uncovered that expression of miR-448 was lower in glioma cell lines (U87, T98, U251, and U373) than in NHA cells (Figure 1A). Meanwhile, CTTN was higher expressed in glioma cell lines (U87, T98, U251, and U373) than in NHA cells (Figure 1B). The finding indicated that miR-448 might be involved in the development of glioma. Thus, U87 and U373 cell lines were chosen to perform our study. According to the expression of miR-448 in glioma cell lines, U87 cells were transfected with miR-448 mimics; U373 cells were transfected with miR-448 inhibitor. The transfection effects were confirmed as shown in Figure 1C and 1D.

### MiR-448 Could Regulate Cell Proliferation

MTT assay was used to detect cell viability. The results demonstrated that the viability of U87

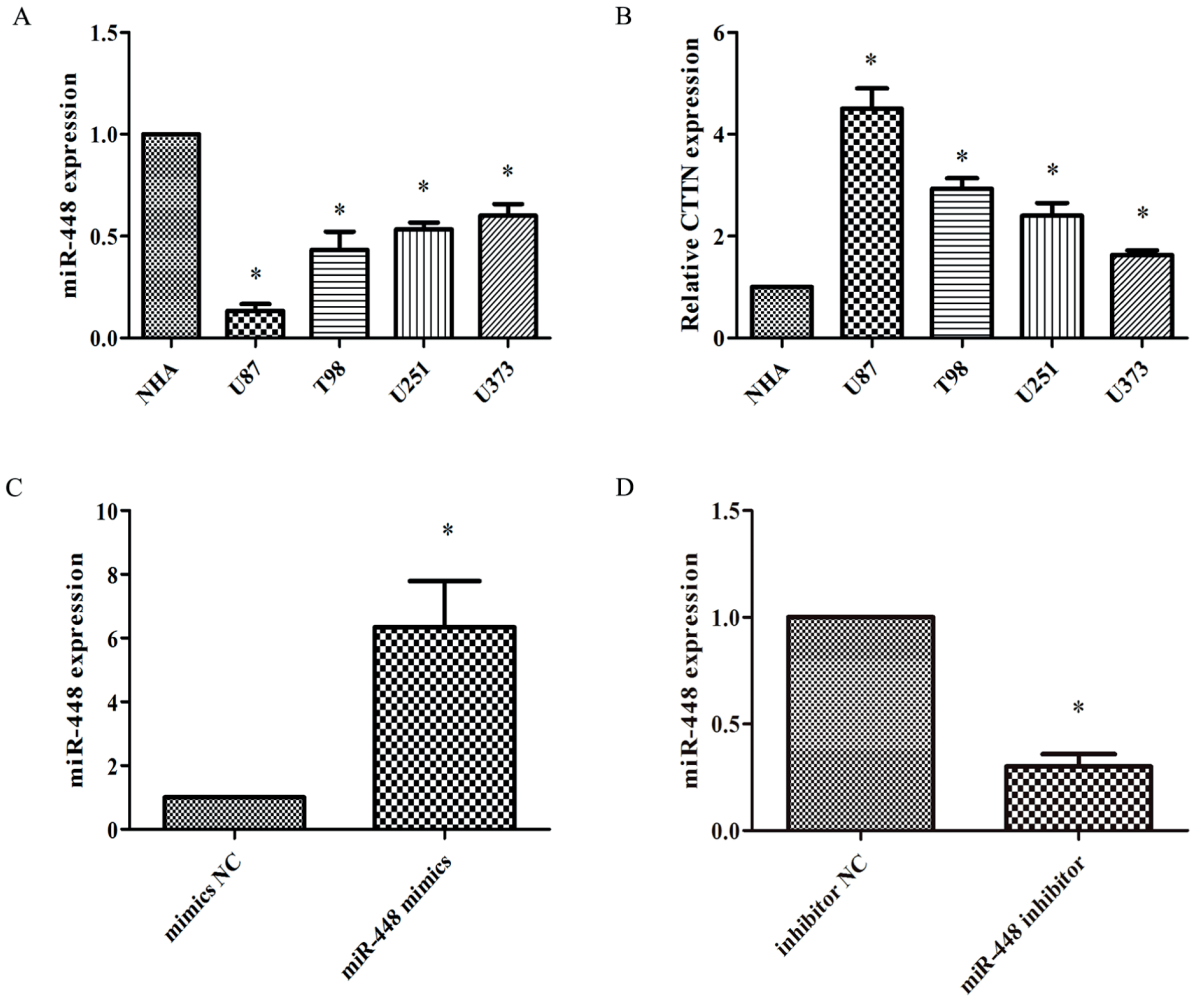


**Figure 1.** Downregulated miR-448 and overexpressed CTTN were detected in glioma cells. ***A,*** miR-448 was lower in glioma cell lines (U87, T98, U251 and U373) than in the cell line (NHA). ***B,*** CTTN was higher in glioma cell lines (U87, T98, U251 and U373) than in the cell line (NHA). ***C,*** U87 cells were transfected with miR-448 mimics. ***D,*** U373 cells were transfected with miR-448 inhibitor. The transfection effects were confirmed by using qRT-PCR method. $p<0.05$ *

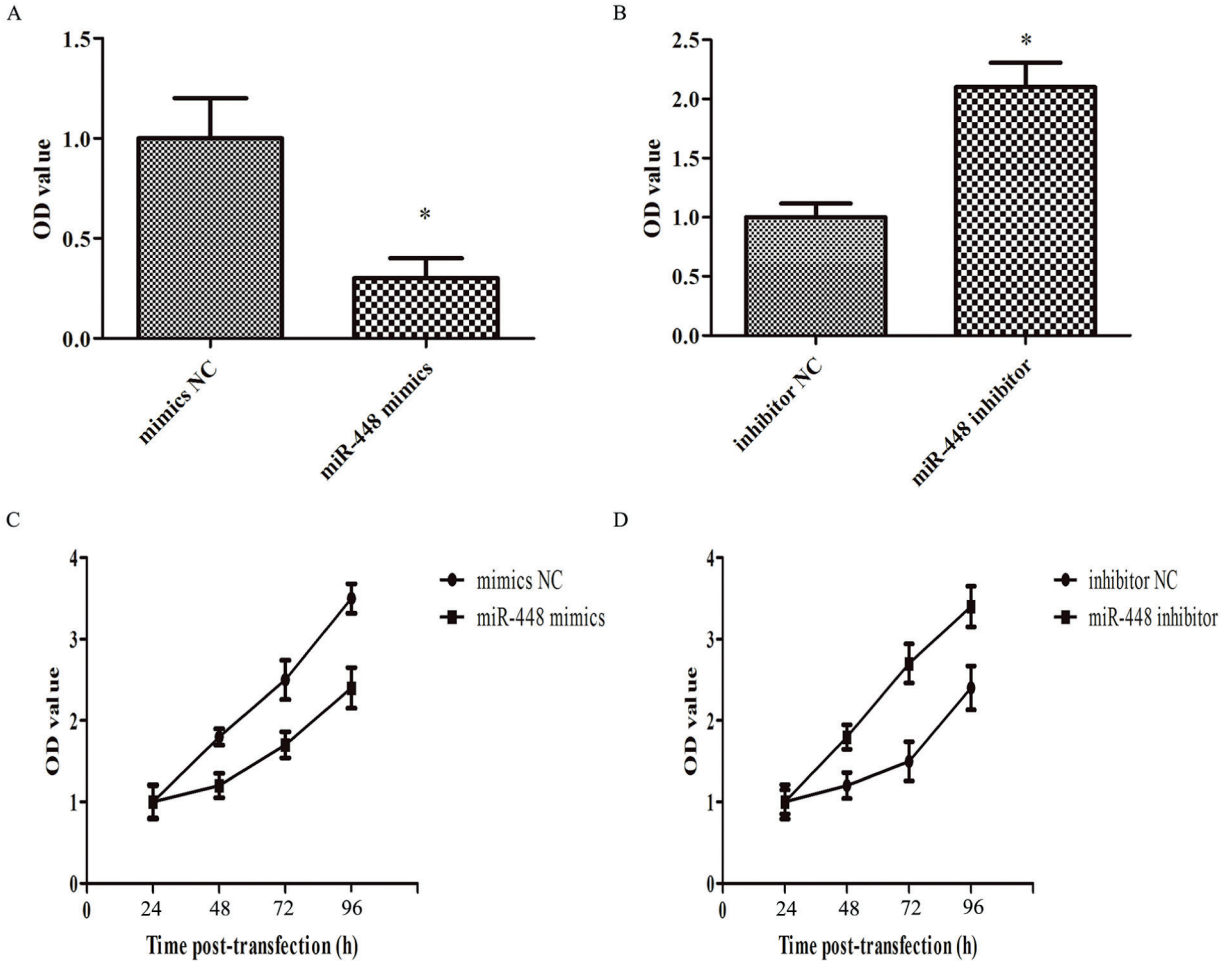


**Figure 2.** MiR-448 could regulate cell proliferation. ***A*** (U87) and ***B*** (U373), Cell proliferation was detected by MTT. ***C*** (U87) and ***D*** (U373): Cell proliferation was detected by CCK8. $p<0.05$ *

cells transfected with miR-448 mimics was significantly decreased compared to the cells transfected with mimics NC ($p<0.05$, Figure 2A). On the contrary, the viability of U373 cells transfected with miR-448 inhibitor was significantly elevated compared to the cells transfected with inhibitor NC ($p<0.05$, Figure 2B).

The CCK-8 assay was also used to detect the cell proliferation. The results showed that cell proliferative ability of U87 cells transfected with miR-448 mimics was significantly decreased as compared with the cells transfected with mimics NC ($p<0.05$, Figure 2C). Conversly, cell proliferative ability of U373 cells transfected with miR-448 inhibitor was significantly elevated as compared with the cells transfected with inhibitor NC ($p<0.05$, Figure 2D).

### *MiR-448 Could Influence Cell Apoptosis and Cell Cycle*

Cell apoptotic rate was measured via using flow cytometry analysis. The results revealed that the apoptotic rate of U87 cells transfected with miR-448 mimics was markedly increased compared to mimics NC ($p<0.05$, Figure 3A). A reverse effect was showed in U373 cells transfected with miR-448 inhibitor as compared with the cells transfected with inhibitor NC ($p<0.05$, Figure 3B).

Furthermore, cell cycle was also detected by using flow cytometry analysis. In U87 cells transfected with miR-448 mimics, the proportion of G0/G1-phase cells was significantly enhanced, whereas the proportion of S-and G2/M-phase cells was significantly lessened (Figure 3C). These results implied that the upregulation of

miR-448 led to cell cycle arrest at the G1/S phase, implying that miR-448 inhibited the cell cycle progression of glioma. The effect was opposite in U373 cells transfected with miR-448 inhibitor (Figure 3D).

### CTTN Was Negatively Regulated by miR-448

The two bioinformatics prediction software (miRNADB and TARGETSCAN) indicated that CTTN was a potential gene of miR-448 (Figure

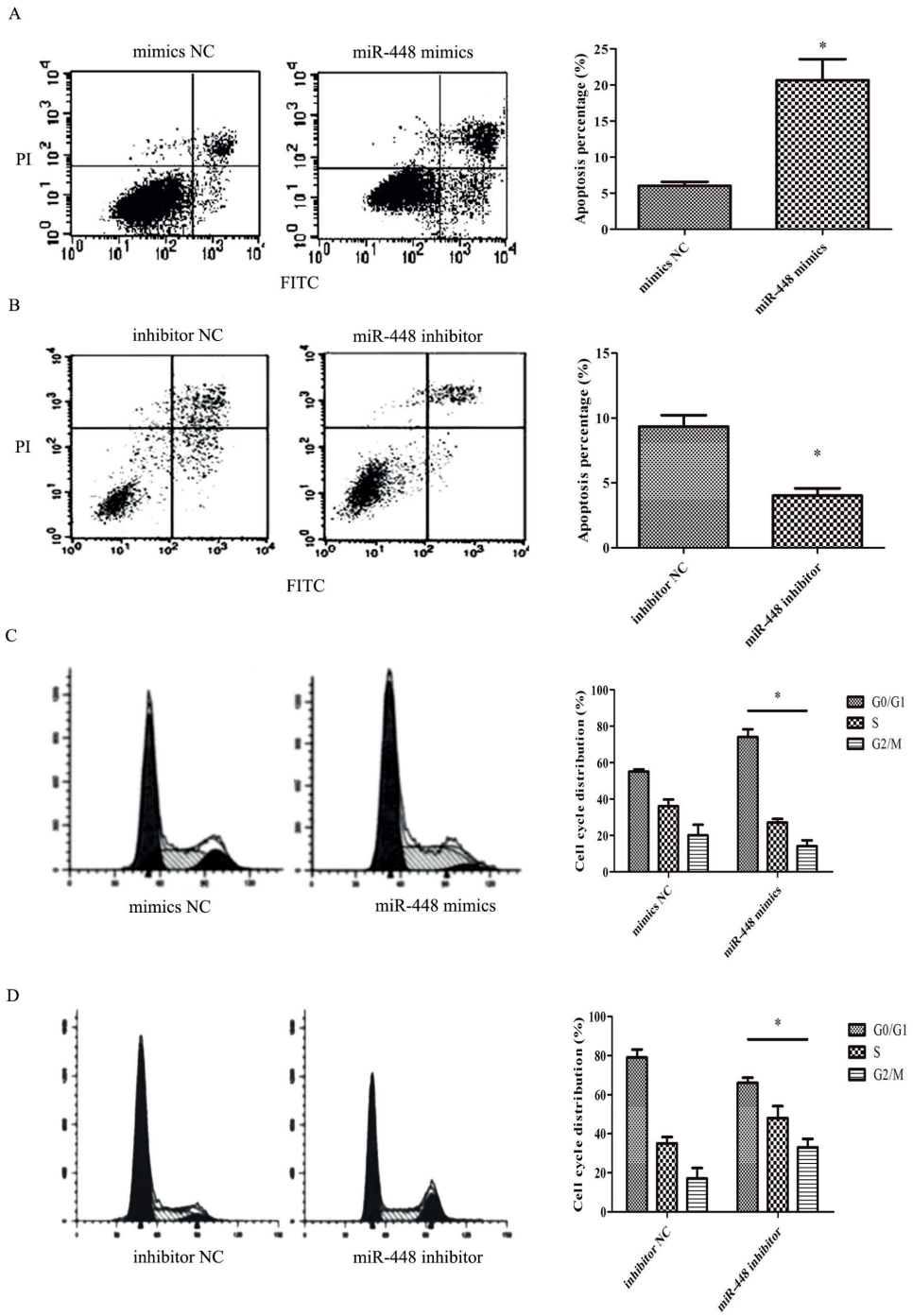


**Figure 3.** MiR-448 could influence cell apoptosis and cell cycle. ***A,*** (U87) and ***B,*** (U373): Cell apoptotic rate was measured via using flow cytometry method. ***C,*** (U87) and ***D,*** (U373): Cell cycle was detected by using flow cytometry method. $p<0.05$ *

4A), and the predictive binding site was shown in Figure 4B. Furthermore, to identify this regulatory mechanism, we applied a luciferase reporter assay. The results indicated that the reporter activity was only lessened in cells transfected with miR-448 mimics and CTTN 3`UTR-wild type (Figure 4C). qRT-PCR analysis demonstrated that CTTN expression was decreased in U87 cells transfected with miR-448 mimics, and was increased in U373 cells transfected with miR-448 inhibitor (Figure 4D and F).

## Discussion

miRNA is an endogenous non-coding small RNA that can regulate approximately 30% human genes expression through mRNA translation and degradation[17]. Dysregulation of miRNAs is present in most human tumors, indicating that miRNAs play a role in the occurrence and development of different tumors[18]. miRNAs can be acted as tumor promotors by inhibiting tumor suppressor genes, and also can function as tumor inhibitors by suppressing tumor oncogenes. Thus, miRNAs in different cell types and genes expression patterns can play both carcinogenic and anti-cancer effects.

In this study, we demonstrated that downregulated miR-448 and overexpressed CTTN were detected in glioma cells. Both MTT and CCK8 assays found that cell proliferation of U87 cells transfected with miR-448 mimics was significantly downregulated as compared with the cells transfected with mimics NC. Conversely, cell proliferation of U373 cells transfected with miR-448 inhibitor was significantly upregulated as compared with the cells transfected with inhibitor NC, which indicated that miR-448 could regulate cell proliferation. miR-448 also could influence cell apoptosis. Up-regulation of miR-448 led to cell cycle arrest at the G1/S phase; the effect was opposite in U373 cells transfected with miR-448 inhibitor, which implying that miR-448 inhibited the cell cycle progression of glioma.

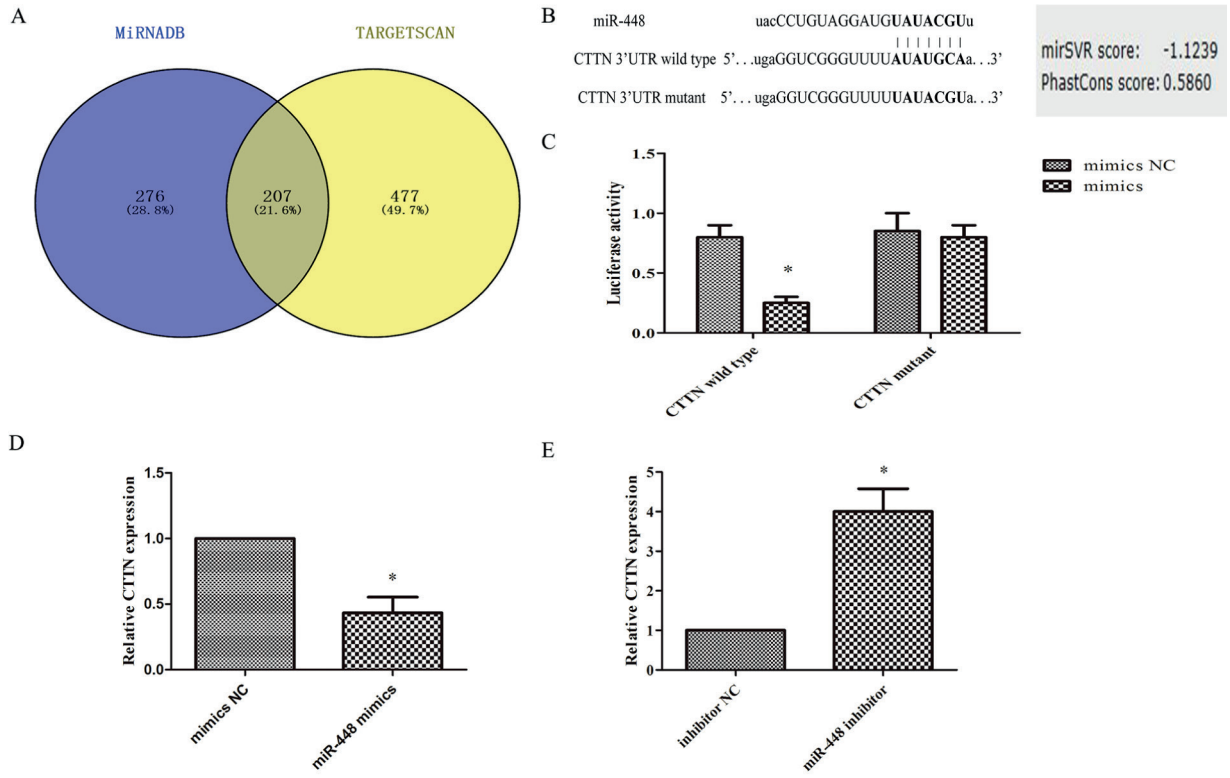


**Figure 4.** CTTN was negatively regulated by miR-448. ***A,*** Venny analysis of the two bioinformatics prediction softwares (miRNADB and TARGETSCAN) indicated that CTTN was a potential gene of miR-448. ***B,*** The predictive binding site was shown. ***C,*** The luciferase reporter assay found that the reporter activity was only lessened in cells transfected with miR-448 mimics and CTTN 3`UTR-wild type. ***D,*** (U87) and ***E,*** (U373): qRT-PCR analysis demonstrated that CTTN expression was decreased in U87 cells transfected with miR-448 mimics, and was increased in U373 cells transfected with miR-448 inhibitor. $p<0.05$ *

CTTN was a potential target gene of miR-448, CTTN expression was decreased in U87 cells transfected with miR-448 mimics, and was increased in U373 cells transfected with miR-448 inhibitor, which suggested that CTTN was negatively regulated by miR-448.

CTTN (cortactin) is overexpressed in a variety of cancers[19], and the aberrant regulation of this gene contributes to tumor cell invasion, metastasis, and proliferation[20]. CTTN could enhance cell proliferation via the EGFR-MAPK pathway in colorectal cancer[21]. Overexpression of CTTN was related to advanced stage and reduced survival in osteosarcoma[22]. CTTN was able to be suppressed by MTSS1 that inhibiting cell migration and invasion in glioblastoma[23]. CTTN was increased in glioma tissues, and downregulated CTTN by siRNA could promote the migration and invasion of glioma cells[24].

## Conclusions

At present, the treatment of glioma by miRNAs has become a research hotspot. Although most studies are still *in vitro* and in animal experiments, there are still some problems that need to be solved if they are applied clinically. However, studies have shown that miRNAs can not only prevent the occurrence of glioma from occurring mechanisms, but also increase the sensitivity of gliomas to radiotherapy and chemotherapy, and can effectively treat glioma and increase survival rate. Therefore, miRNAs researches will have a broad prospect and will open up new perspectives for humans to overcome glioma.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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