

# MiR-532-5p acts as a tumor suppressor and inhibits glioma cell proliferation by targeting CSF1

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**Abstract. – OBJECTIVE:** Recent studies have discovered a class of micro-RNAs (miRNAs), which are dysregulated in various tumors and associated with carcinogenesis. In our research, we aim to uncover the molecular functions of miR-532-5p in glioma development.

**PATIENTS AND METHODS:** Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to detect miR-532-5p expression in 48 glioma samples and 4 glioma cell lines. The Pearson's Chi-square test was used to determine the association of miR-532-5p expression with several clinicopathological indexes in glioma patients. Besides, cell proliferation assay, colony formation assay, and Ethynyl deoxyuridine (EdU) incorporation assay were performed to explore *in vitro* effects of miR-532-5p on glioma cells. Furthermore, the interaction between miR-532-5p and CSF1 in glioma was studied by performing Western blot assay and Dual-Luciferase Reporter Gene Assay.

**RESULTS:** Downregulated miR-532-5p expression was observed in glioma tissues compared with adjacent normal samples. MiR-532-5p expression was associated with the KPS score and tumor grading in glioma patients. Moreover, cell proliferation of glioma was inhibited by overexpression of miR-532-5p *in vitro*. Furthermore, CSF1 was a target of miR-532-5p. In glioma, overexpression of miR-532-5p was downregulated at mRNA and protein level *in vitro*. Besides, the expression of CSF1 in glioma tissues was negatively related to the expression of miR-532-5p.

**CONCLUSIONS:** Malignant phenotypes of glioma cells were remarkably suppressed through the overexpression of miR-532-5p. MiR-532-5p/CSF1 axis is identified as a new therapeutic intervention for the treatment of glioma.

**Key Words:** Glioma, miR-532-5p, CSF1.

## Introduction

Glioma is one of the most ordinary subtypes of malignant intracranial cancers in adults which is also one of the most lethal and aggressive types

of cancers in the world<sup>1</sup>. Although great progress has been made in the standard treatment for glioma, the satisfactory outcomes for patients remain dismal<sup>2</sup>. The median survival of glioma is approximately 15 months and five-year survival is extremely poor<sup>3</sup>. Glioma has heterogeneous characteristics, posing a huge challenge on the current treatment regimen, it is urgent to demonstrate the mechanisms underlying the development of glioma and to explore the potential therapeutic targets for human glioma.

Micro RNA (miRNAs) are known as small non-coding RNA molecules with 18-22 nucleotides in length. MiRNAs are able to regulate cell proliferation, apoptosis, and differentiation by inducing mRNA degradation or repressing translation<sup>4</sup>. Recently, numerous studies have indicated the vital functions of miRNAs in various diseases, including tumorigenesis. By dysregulating miR-141, miR-143, miR-145, miR-147, miR-154, miR-155, miR-197, miR-200c, miR-21, miR-22, miR-23a, miR-23b, miR-24, miR-25, miR-27a, miR-27b, miR-29a, miR-29b, miR-31, miR-32, miR-34a, miR-34b, miR-34c, miR-36, miR-37, miR-39, miR-41, miR-42, miR-43, miR-44, miR-45, miR-46, miR-47, miR-48, miR-49, miR-50, miR-51, miR-52, miR-53, miR-54, miR-55, miR-56, miR-57, miR-58, miR-59, miR-60, miR-61, miR-62, miR-63, miR-64, miR-65, miR-66, miR-67, miR-68, miR-69, miR-70, miR-71, miR-72, miR-73, miR-74, miR-75, miR-76, miR-77, miR-78, miR-79, miR-80, miR-81, miR-82, miR-83, miR-84, miR-85, miR-86, miR-87, miR-88, miR-89, miR-90, miR-91, miR-92, miR-93, miR-94, miR-95, miR-96, miR-97, miR-98, miR-99, miR-100, miR-101, miR-102, miR-103, miR-104, miR-105, miR-106, miR-107, miR-108, miR-109, miR-110, miR-111, miR-112, miR-113, miR-114, miR-115, miR-116, miR-117, miR-118, miR-119, miR-120, miR-121, miR-122, miR-123, miR-124, miR-125, miR-126, miR-127, miR-128, miR-129, miR-130, 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In this study, we found out that miR-532-5p, a newly discovered microRNA in cancers, was remarkably downregulated in glioma samples and was associated with clinicopathological indexes in glioma patients. Moreover, functional experiments revealed that miR-532-5p depressed cell proliferation in glioma. Furthermore, we discovered the target proteins of miR-532-5p in glioma.

## Patients and Methods

### Clinical Samples

Glioma tissues and paired adjacent normal tissues were collected from 40 glioma cases and pre-

served in liquid nitrogen. Their clinicopathological characters were analyzed by two pathologists. This study was approved by the Ethics Committee of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. The signed written informed consents were obtained from all participants before the study.

### Cell Lines

Four glioma cell lines (U251, U87, T98, and U373), and the normal human astrocyte 1800 cell line, were offered by the Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum FBS (FBS; Gibco, Rockville, MD, USA) was used for cell culture.

### Lentiviral Virus Transfection

Lentiviral virus targeting miR-532-5p was compounded by Genepharma (Suzhou, China). The glioma cells, grown to 70%-80% confluence, were transfected with miR-532-5p lentiviruses (miR-532-5p) or empty vector using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA).

### RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was extracted from glioma or glioma cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNeasy RNeasy Spin Kit (Qiagen, Crawfordsville, IN, USA) and RNeasy RNeasy Spin Kit (Qiagen, Crawfordsville, IN, USA) were used for synthesizing cDNAs. The primers were shown as follows: miR-532-5p, forward: 5'-GGCCATGCCTTGAGTGTA-3' and reverse: 5'-CAGG-GTCCGAGGTA-3'; U6, forward: 5'-TGC-GGGTGCTCC-3' and reverse: 5'-CCAGTGGGT-3';  $\beta$ -actin, forward: 5'-AATC-3' and reverse: 5'-CAGAGGCT-3'. RT-PCR system was prepared as follows: 10  $\mu$ L of forward primer, 0.4  $\mu$ L of reverse primer, 4  $\mu$ L of ROX Reference Dye, 1  $\mu$ L of RT-Stratagene, 1  $\mu$ L of double-distilled water, and 1  $\mu$ L of All-in-One™ qPCR Mix (GenScript, Rockville, MD, USA). The system was reacted in ABI PRISM 7000 Fluorescent Quantitative System (Applied Biosystems, Foster City, CA, USA).  $\beta$ -actin was used as an

internal control. The relative expression was calculated by performing the  $2^{-\Delta\Delta CT}$  method.

### Cell Counting Kit-8 (CCK-8) Assay

The cell proliferation was measured by CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, 1000 cells/mL CCK-8 was added at each time point (0, 24, 48, and 72 h). OD450 was measured using Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) after cells incubation for 1 h.

### Colony Formation Assay

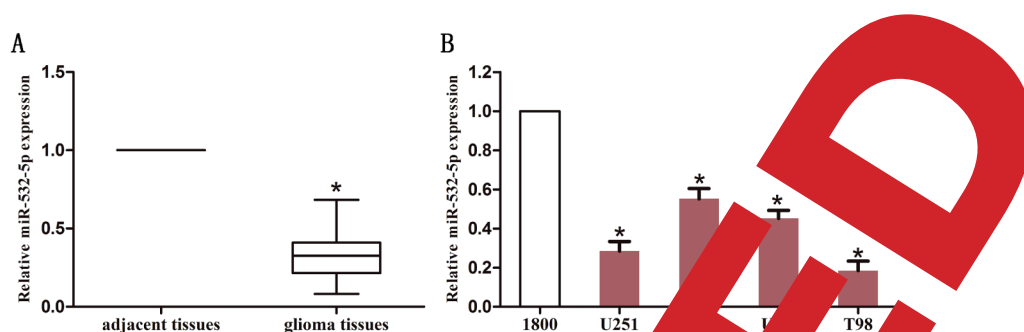
To determine long-term effect of miR-532-5p on cell proliferation, the colony formation assays were conducted. 500 cells/well were inoculated in a 6-well plate and the culture medium was replaced every day. 7 day later, the colonies were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet. The colonies were photographed and counted.

### Ethyl-3-(3-Dimethylamino)propyl Carbodiimide Cross-Linking Reagent (EDU) Incorporation Assay

EDU Apollo *in vitro* Imaging kit (Ribobio, Guangzhou, China) was used in this study. Briefly, the cells ( $6 \times 10^3$ /well) were cultured in 96-well plates after transfection. After incubation with 50  $\mu$ M EdU labeling medium for 2 h at 37°C, the cells were stained in the anti-EdU working solution. Hoechst33342 was used to label cell nuclei. EdU-positive cells were observed by a fluorescent microscope (Olympus, Tokyo, Japan).

### Western Blot Analysis

The glioma cells were lysed using radioimmunoprecipitation assay (RIPA) protein extraction reagent (Beyotime, Beijing, China) supplemented with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF, Roche, Basel, Switzerland). The Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was utilized to detect concentration. The protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody of CSF1 (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight.  $\beta$ -actin (Cell Signaling Technology, Danvers, MA, USA) was utilized as an internal control. The membrane was incubated with the secondary antibody after rinsing with the buffer solution (TBST). Enhanced chemilumines-



**Figure 1.** The expression level of miR-532-5p decreased in the glioma tissues and cells. The miR-532-5p expression significantly decreased in the glioma tissues compared with the adjacent tissues. The expression of miR-532-5p relative to  $\beta$ -actin was determined in the human glioma cell lines and normal human fibrocyte 1800 cells by RT-qPCR. The data are presented as the mean  $\pm$  standard error of the mean. \* $p < 0.05$ .

cence (ECL) was used to expose the protein bands on the membrane.

**Dual-Luciferase Reporter Gene Assay**

CSF1 3'-untranslated region (3'-UTR) was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3'-UTR. The site-directed mutagenesis of the miR-532-5p binding site in CSF1 3'-UTR was performed by quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as mutant (MUT) 3'-UTR. Then, we made the transfection of WT-3'-UTR, MUT-3'-UTR and negative control or miR-532-5p for 48 h. Then, the luciferase assay was conducted on the Dual-Luciferase Reporter Gene Assay System (Promega, Madison, WI, USA).

**Statistical Analysis**

The paired samples *t*-test was used to compare the miR-532-5p expression in glioma tumor tissues

and paired normal tissues. The independent samples *t*-test were used for functional assays. Pearson's Chi-square test was used to determine the association of miR-532-5p expression with clinicopathological indexes in glioma patients. The Statistical Product and Service Solutions (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical tests.  $p < 0.05$  was regarded as statistically significant.

**Results**

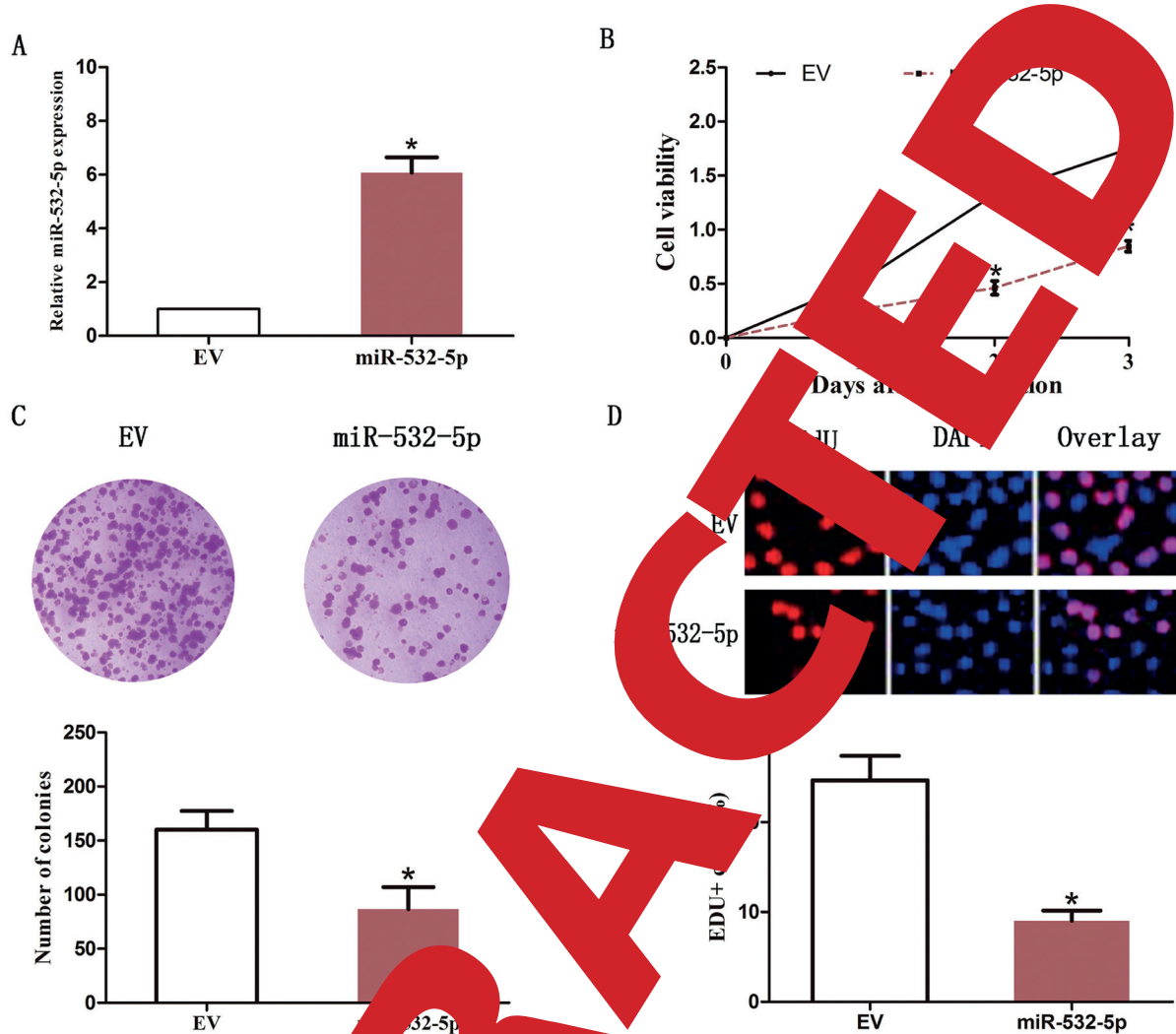
**Expression Level of MiR-532-5p Was Lower in Glioma Tissues and Cells**

MiR-532-5p expression was measured by RT-qPCR in 40 glioma tissues and four glioma cell lines. MiR-532-5p was lowly expressed in glioma tissues compared to adjacent tissues (Figure 1A). The association between miR-532-5p expression with the

**Table I.** Correlation between miR-532-5p expression and clinicopathological characteristics in glioma patients.

Characteristics	Patients	Expression of miR-532-5p		p-value
		High group	Low group	
Total	40	17	23	
Age (years)				0.622
$\leq 50$	15	6	9	
$> 50$	25	12	13	
Gender				0.821
Male	17	9	8	
Female	23	13	10	
WHO grade				0.019
II	22	13	9	
III	18	4	14	
Survival (months)				0.002
$\geq 90$	17	12	5	
$< 90$	23	5	18	

$p < 0.05$  is considered as statistically significant.



**Figure 2.** The overexpression of miR-532-5p inhibited the glioma cell proliferation. **A**, MiR-532-5p expression in glioma cells transfected with the empty vector (EV) or miR-532-5p lentivirus (miR-532-5p) was detected by RT-qPCR.  $\beta$ -actin was used as an internal control. **B**, CCK-8 assay showed that overexpression of miR-532-5p significantly repressed cell proliferation in glioma cells. **C**, The colony formation assay showed that the overexpression of miR-532-5p significantly repressed the cell growth ability of glioma cells (magnification  $\times 40$ ). **D**, EdU assay showed that the EdU-positive glioma cells were significantly reduced via overexpression of miR-532-5p (magnification  $\times 200$ ). The results represent the average of three independent experiments (mean  $\pm$  standard error). \* $p < 0.05$ .

demographic and clinicopathological characteristics of glioma patients were then assessed (Table I). MiR-532-5p expression was significantly associated with the tumor size ( $p = 0.002$ ) and tumor grade ( $p = 0.019$ ) but not associated with the age and gender of glioma patients ( $p > 0.05$ ). Besides, the miR-532-5p level was significantly lower in glioma tissues than that in normal human astrocyte 180 cell line.

#### Inhibition of Cell Proliferation by Overexpression of MiR-532-5p

T98 glioma cells were transfected with miR-532-5p lentivirus or empty vector. 48 h later, miR-

532-5p expression was analyzed by RT-qPCR (Figure 2A). The changes in glioma cell proliferation were monitored by CCK-8 assay (Figure 2B), colony formation assay (Figure 2C), and EdU assay (Figure 2D), respectively. The inhibited cell proliferation was observed in glioma cells after the overexpression of miR-532-5p.

#### Expression Level of CSF1 Was Lower in Glioma Tissues and Cells

To explore the possible downstream targets associated with miR-532-5p, CSF1 was selected to be the potential targets by analyzing in StarBase v2.0. The binding sequence of miR-532-5p in CSF1 was

shown in Figure 3A. The expression level of CSF1 was detected in glioma tissues and cells. CSF1 expression was significantly higher in glioma tissues compared with that in adjacent tissues (Figure 3B). Similarly, CSF1 expression was remarkably higher in glioma cells compared with normal human astrocyte 1800 cell line (Figure 3C).

### Overexpression of MiR-532-5p Downregulated CSF1 in Glioma

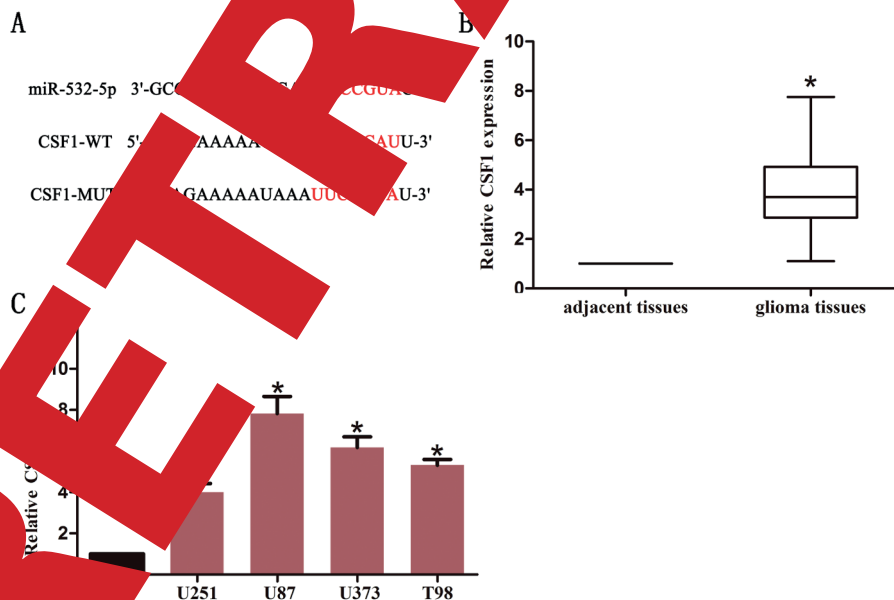
RT-qPCR results further demonstrated that CSF1 expression was downregulated after glioma cells were transfected with miR-532-5p lentivirus (Figure 4A). Western blot analysis results also revealed that the protein level of CSF1 was downregulated after glioma cells transfection with miR-532-5p lentivirus (Figure 4B). The Luciferase activity was significantly inhibited via co-transfection of CSF1-WT and miR-532-5p (Figure 4C). The results of linear correlation analysis showed that in glioma tissues, the expression of CSF1 was negatively correlated to miR-532-5p expression (Figure 4D).

rious side effects of traditional for cancers, molecular-targeted therapy has well concerned<sup>9</sup>. Studies have proved that miRNAs participate in tumorigenesis and development of glioma. The cell migration and cell invasion of glioma are promoted by miRNAs, while they are suppressed by miR-126<sup>11</sup>. The proliferation of glioma is also suppressed by miR-599<sup>12</sup>. Moreover, the aggressiveness properties of glioma could be inhibited by miR-10a<sup>13</sup>.

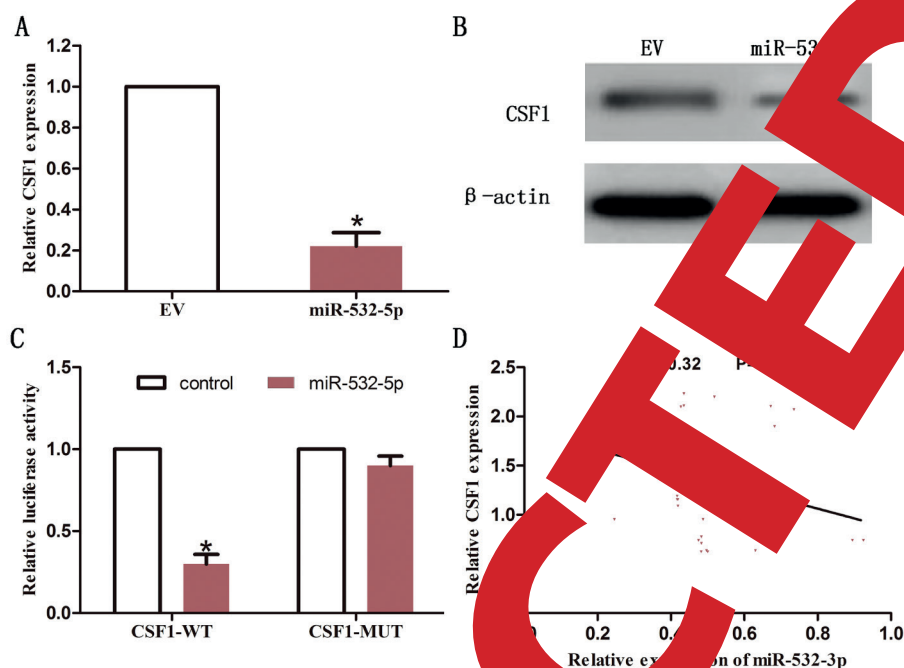
MiR-532-5p is a newly discovered microRNA which has been identified as closely related to the development and carcinogenesis of several tumors. In addition, by activating the HMGB3/Wnt/ $\beta$ -catenin signaling pathway, low expression of miR-532-5p promotes cell proliferation and invasion in bladder cancer<sup>14</sup>. MiR-532-5p participates in the regulation of molecular pathogenesis and carcinogenesis in renal cell carcinoma<sup>15</sup>. By inhibiting CXCL2, miR-532-5p serves as a tumor suppressor in hepatocellular carcinoma via inhibiting cell proliferation and cell metastasis<sup>16</sup>. Our study showed that the expression of miR-532-5p was downregulated in both glioma tissue and cells. MiR-532-5p expression was associated with glioma core and tumor grade of glioma. Furthermore, after miR-532-5p was overexpressed, the ability of cell growth was suppressed. These

### Discussion

Currently, a lack of efficient therapy for gliomas is a huge challenge worldwide. To avoid the side effects of cell growth is suppressed. These



**Figure 3** The expression level of CSF1 increased in glioma tissues and cell lines. **A**, The binding sequence of miR-532-5p in CSF1. **B**, CSF1 expression significantly upregulated in the glioma tissues compared with the adjacent tissues. **C**, The expression level of CSF1 in glioma cell lines.  $\beta$ -actin was determined in the human glioma cell lines and normal human astrocyte 1800 cell line by RT-qPCR. The data are presented as the mean  $\pm$  standard error. \* $p < 0.05$ .



**Figure 4.** Interaction between CSF1 and miR-532-5p in glioma. **A**, The expression of CSF1 in miR-532-5p cells significantly decreased compared with the empty control cells in the glioma cells. **B**, The protein expression of CSF1 decreased after the overexpression of miR-532-5p in glioma cells. **C**, The transfection of miR-532-5p and CSF1-WT strongly decreased the Luciferase activity, while the co-transfection of miR-532-5p and CSF1-MUT did not change the Luciferase activity. **D**, The linear correlation between the expression levels of CSF1 and miR-532-3p in glioma tissues. The results represent the average of three independent experiments. \* $p < 0.05$ .

data indicated that miR-532-5p functions as a tumor suppressor and inhibited tumor progression of glioma. miR-532-5p is a promising biomarker for glioma.

CSF1 is a cytokine for macrophages and microglia which has been identified to be a candidate oncogene in high-grade gliomas induced by the Sleeping Beauty system<sup>17</sup>. Moreover, several researchers<sup>18,19</sup> have shown that CSF1 is over-expressed in glioblastoma. In this experiment, the outcome of RT-qPCR and Western blot indicated that CSF1 was downregulated after miR-532-5p overexpression *in vitro*. Moreover, a negative correlation was observed between CSF1 and miR-532-5p expression in glioma tissues. Further experiments showed that CSF1 was identified as the target protein of miR-532-5p. The results above reveal that miR-532-5p may realize its function in glioma progression via targeting CSF1.

### Conclusions

We suggest miR-532-5p as a new biomarker in the development of glioma. MiR-532-5p is vital in the car-

### Conflicts of interest

The authors declare no conflicts of interest.

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