European Review for Medical and Pharmacological Sciences 2019; 23: 8964-8970

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

Y.-P. WANG, J. LIU, D. LIU, X.-D. WANG, A.-M. BIAN, D.-Z. FANG, X.-B. HUI

Department of Neurosurgery, The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University, Huaian, China

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, and was downregulated by overexpression of miR-532-5p, which was decreased regulated at mRNA and protein levels *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:
MicroRNA, MiR-532-5p, Glioma, 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[1]. Although great progress has been made in the standard treatment for glioma, the satisfactory outcomes for patients remain dismal[2]. The median survival of glioma is approximately 15 months and the five-year survival is extremely poor. Glioma is heterogeneous characteristics, posing a huge challenge on the current treatment. Thus, it is urgent to demonstrate the mechanisms underlying the development of glioma and find out the potential therapeutic targets for human glioma.

MicroRNAs (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[5]. Recently, numerous studies have indicated critical functions of miRNAs in various diseases, including tumorigenesis. By dysregulating KLF4/TX2, miR-644a is downregulated in esophageal squamous cell carcinoma and promotes the aggressiveness and stemness phenotype[6]. By targeting CD15, the miR-199b-5p expression is remarkably downregulated in patients with metastatic medulloblastoma[7]. By regulating the expression of STAT6, miR-1207-5p promotes cell proliferation in breast cancer[8].

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-

Corresponding Author: Xiaobo Hui, MD; e-mail: huixiaobo2003113@163.com

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 lentivir[illegible] (miR-532-5p) or empty vector using Li[illegible] amine 3000 Transfection Reagent (Inv[illegible], Carlsbad, CA, USA).

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

The total RNA was extrac[illegible] fro[illegible] or glioma cells using TRI[illegible]eage[illegible] rogen, Carlsbad, CA, USA). [illegible]rtAid™ First Strand complemen[illegible] de[illegible] nucleic acid (cDNA) Sy[illegible]is Kit ([illegible]s, Hanover, NH, USA) [illegible]ed for synthe[illegible]ing cDNAs. The primer[illegible] were shown as follows: miR-532-[illegible] forw[illegible]CGGCCAT-GCCTTGAGTGTA-3′ and reve[illegible]GCAGG-GTCCGAGGTA[illegible]C-3′; U6, forwa[illegible] 5′-TGC-GGGTGCTCG[illegible]TCG[illegible]AGC-3′ and reverse: 5′-CCAGTG[illegible]GGT[illegible]GAGGT-3′; β-actin, forward [illegible]AATC[illegible]CAGAGGCT-3′ and reverse [illegible]CAC[illegible]AGTTGGAAAT-GC-3′. RT-qPCR sy[illegible]L) was prepared as follow[illegible] of forw[illegible]rimer, 0.4 μL of revers[illegible]me, 0.4 μL of ROX Reference Dye, 1 μL of [illegible]t-Stra[illegible] μL of double-distilled w[illegible]d[illegible] μL of All-in-One™ qPCR Mix (Gen[illegible], Rockville, MD, USA). The system was reac[illegible] ABI PRISM 7000 Fluorescent Quantitative [illegible] System (Applied Biosystems, Foster City, CA, USA). β-actin was used as an internal control. The relative ex[illegible]alculated by performing the $2^{-\Delta\Delta}$[illegible]ethod.

Cell Counting Kit-8 ([illegible] Assay

The cell proliferation wa[illegible]red b[illegible]CK-8 (Beyotime Institut[illegible]f Biote[illegible]hang-hai, China). Briefly[illegible]ng/mL CCK[illegible] added at each time poin[illegible], 24, [illegible]and 72 h. OD450 was measured u[illegible]Spec[illegible]hotom[illegible]er (Thermo Fisher Scientific[illegible]MA, [illegible]) after cells incubation f[illegible]l h.

Colony [illegible]nation Assay

To d[illegible]ng-term effect of miR-532-5p on cel[illegible]blife[illegible]e colony formation assays were [illegible]onducted. 5[illegible]/well cells were inocula[illegible]a 6-well plate a[illegible]the culture medium was [illegible]ced every day. 7 day later, the colonies were [illegible]d with 75% [illegible]hanol for 30 min and stained [illegible] 0.2% cryst[illegible]olet. The colonies were pho-[illegible]hed and [illegible]ted.

Ethy[illegible]oxyuridine (EdU) Incorporation Assay

[illegible]U Apollo *in vitro* Imaging kit (Ribobio, [illegible]u, China) was used in this study. Brief-[illegible]he cells (6×10^3/well) were cultured in 96-well [illegible]ates after transfection. After incubation with [illegible]0 μM EdU labeling medium for 2 h at 37°C, the [illegible]lls were stained in the anti-EdU working solu-[illegible]on. 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. β-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-

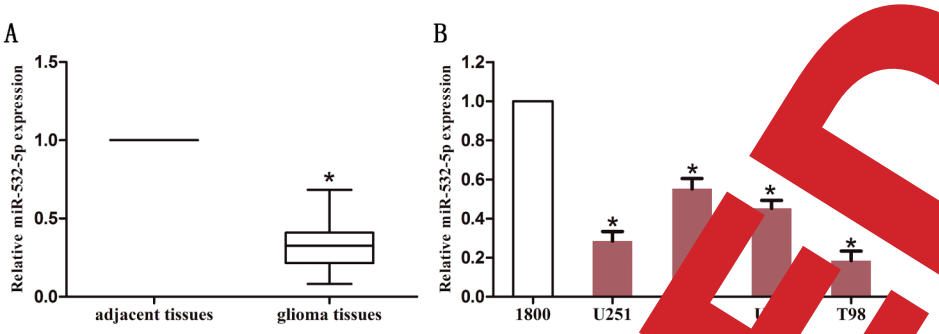


Figure 1. The expression level of miR-532-5p decreased in the glioma tissues and cell lines. A, MiR-532-5p expression significantly decreased in the glioma tissues compared with the adjacent tissues. B, The expression of miR-532-5p relative to β-actin was determined in the human glioma cell lines and normal human astrocyte 1800 cells by RT-qPCR. The data are presented as the mean ± 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. After that, we made the transfection of WT-3′-UTR or 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 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	Pa	Expression of miR-532-5p		*p*-value
		High group	Low group	
Total	40	17	23	
Age (years)				0.622
≤50	15	6	9	
>50	25	12	13	
Gender				0.821
Male	17	9	8	
Fe	23	13	10	
W				0.019
	22	13	9	
	18	4	14	
				0.002
≥90	17	12	5	
<90	23	5	18	

$p<0.05$ is considered as statistically significant.

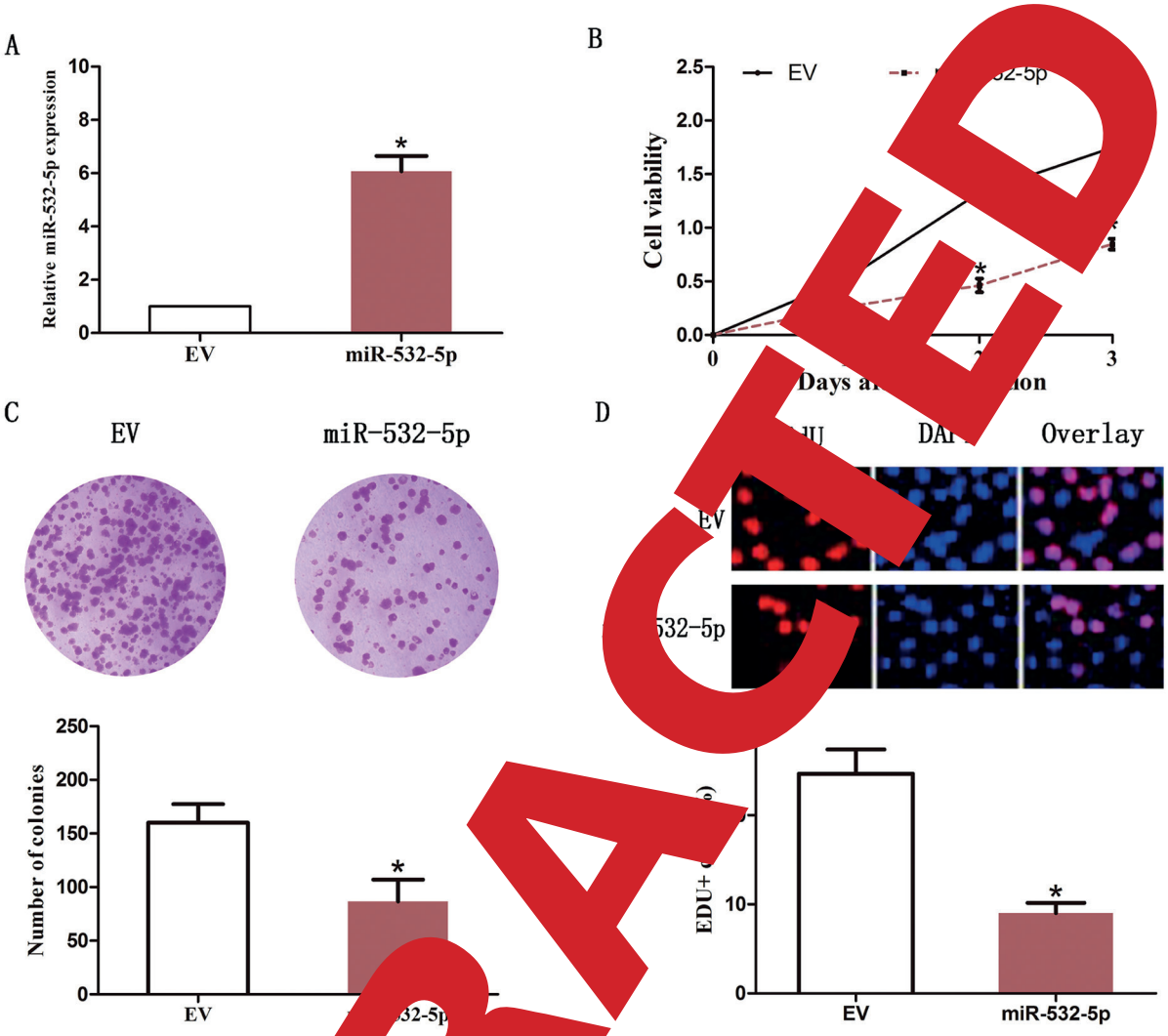


Figure 2. The overexpression of miR-532-5p inhibited the glioma cell proliferation. **A,** MiR-532-5p expression in glioma cells transfected with the empty vector (EV) or miR-532-5p lentivirus (miR-532-5p) was detected by RT-qPCR. β-actin was used as an internal control. **B,** CCK-8 assay showed that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

demographic and clinicopathological characteristics of glioma patients were then assessed (Table I). MiR-532-5p expression was significantly associated with the WHO grade ($p=0.00$) 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 cells than that in normal human astrocyte 18 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).

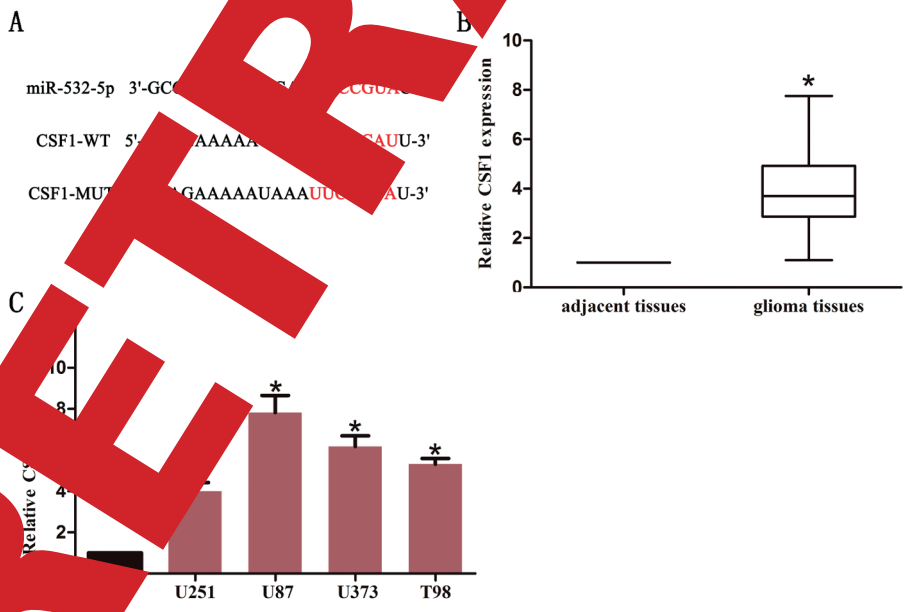
Overexpression of MiR-532-5p Downregulated CSF1 in Glioma

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).

Discussion

Currently, a lack of efficient therapy fo[r] cers is a huge challenge worldwide. To avoi[d] rious side effects of tradition[al] cancers, molecular-targeted th[erapy] has [been] well concerned[9]. Studies have [demonstrated] that [m]iRNAs participate in tumorigen[esis] and develop[ment] of glioma. The cell migrati[on] and cell inv[asion] of glioma are promoted by mi[croRNAs] they are suppressed by [miR]-126[11]. The [p]roliferation of glioma is [also] sup[pressed] by miR-599[12]. Moreover, the [aggressiveness] properties of glioma could be inhib[ited] by miR-30a[13].

MiR-532-5p is a [newly] disco[vered] microRNA which has [been] identi[fied] closely related to the devel[opment] and carc[inogen]esis of several tumors [prevalence], by activating the HMGB3/Wnt/β-[catenin] signaling pathway, low expression of miR-532-5p [promotes] cell proliferation and in[vasion] in bladder [cancer][14]. MiR-532-5p participates in the regulation of molecular pathogenesis [and] oncogenesis in renal cell carcinoma[15]. By inhibiting CXCL2, miR-532-5p serves as a tumor suppressor in h[epatocellular] carcinoma via inhibiting cell p[roli]feration and cell metastasis[16]. Our study [found] that the expression of miR-532-5p was downregulated in both glioma tissue and cells. MiR-532-5p expression was associated with [the] score and tumor grade of glioma. Furthermore, after miR-532-5p was overexpressed, the ability of cell growth was suppressed. These



Figu[re] [e]xpression level of CSF1 increased in glioma tissues and cell lines. **A,** The binding sequence of miR-532-5p in CSF1. **B** [was] significantly upregulated in the glioma tissues compared with the adjacent tissues. **C,** The expression level of CSF1 [normalized] to β-actin was determined in the human glioma cell lines and normal human astrocyte 1800 cell line by RT-qPCR. The data [were] presented as the mean ± standard error. *$p<0.05$.

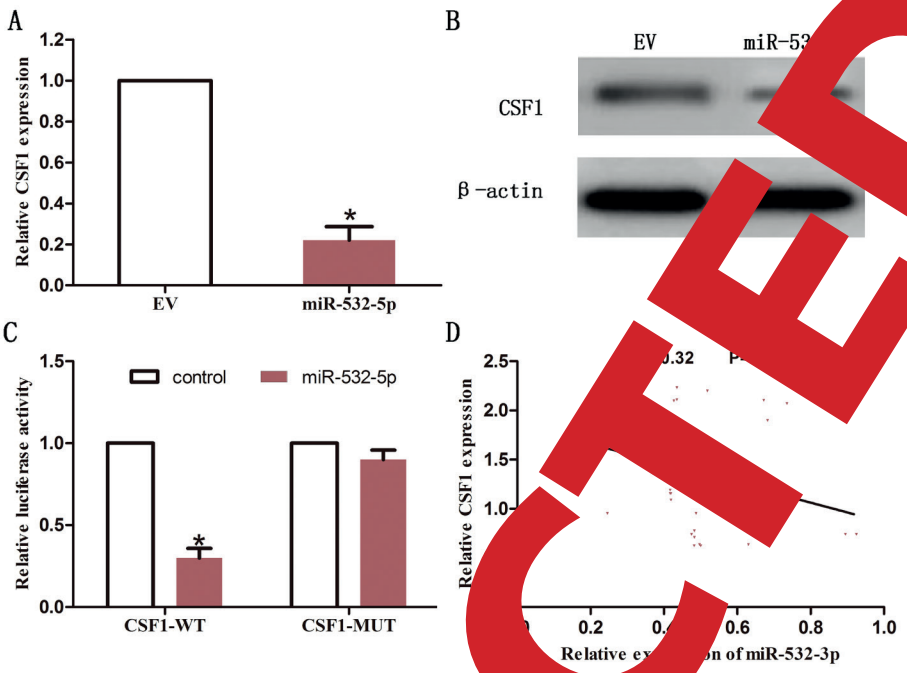


Figure 4. Interaction between CSF1 and miR-532-5p in glioma. **A,** T[illegible]ssion of CSF1 in miR-532-5p cells significantly decreased compared with the empty control cells in the glioma cells. **B,** The protein expression of CSF1 decreased after the overexpression of miR-532-5p in glioma cells.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

data indicated that miR-532-5p fu[illegible]s a tumor suppressor and inhibited [illegible]umo[illegible] of glioma.

CSF1 is a cytokine for ma[illegible]nd microglia which has been ide[illegible]fied t[illegible]didate oncogene in high-grade [illegible]ocytomas [illegible]by the Sleeping Beauty sy[illegible]e[17]. Moreover, [illegible]veral researchers[18,19] have [illegible]hat CSF1 is overexpressed in gliobla[illegible]ma. I[illegible]eriment, the outcome of RT-qPCR and Weste[illegible]ndicated that CSF1 was [illegible]nregulated after [illegible]iR-532-5p overexpressio[illegible]*vitro*. [illegible]oreover, a negative correlation was [illegible]overe[illegible]etwee[illegible] CSF1 and miR-532-5p exp[illegible]ioma[illegible]es. Further experiments sho[illegible]CSF1[illegible]s identified as the target pr[illegible]in of [illegible]. The results above reveal [illegible]iR-532-[illegible]y realize its function in g[illegible]a p[illegible]ession via targeting CSF1.

Conclusions

We sugge[illegible]ew biomarker in the development of glioma. MiR-532-5p is vital in the carcinogenesis of glioma and can be served as a promising biomarker for glioma.

Conflicts of interest

The authors declare no conflicts of interest.

References

1) Sathornsumetee S, Rich JN. New treatment strategies for malignant gliomas. Expert Rev Anticancer Ther 2006; 6: 1087-1104.
2) Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 2004; 70: 217-228.
3) Xu L, Yu QW, Fang SQ, Zheng YK, Qi JC. MiR-650 inhibits the progression of glioma by targeting FAM83F. Eur Rev Med Pharmacol Sci 2018; 22: 8391-8398.
4) Kalpathy-Cramer J, Gerstner ER, Emblem KE, Andronesi O, Rosen B. Advanced magnetic resonance imaging of the physical processes in human glioblastoma. Cancer Res 2014; 74: 4622-4637.
5) Callegari E, D'Abundo L, Guerriero P, Simioni C, Elamin BK, Russo M, Cani A, Bassi C, Zagatti B, Gia-

comelli L, Blandamura S, Moshiri F, Ultimo S, Frassoldati A, Altavilla G, Gramantieri L, Neri LM, Sabbioni S, Negrini M. MiR-199a-3p modulates MTOR and PAK4 pathways and inhibits tumor growth in a hepatocellular carcinoma transgenic mouse model. Mol Ther Nucleic Acids 2018; 11: 485-493.

6) Zhang JX, Chen ZH, Xu Y, Chen JW, Weng HW, Yun M, Zheng ZS, Chen C, Wu BL, Li EM, Fu JH, Ye S, Xie D. Downregulation of microRNA-644a promotes esophageal squamous cell carcinoma aggressiveness and stem cell-like phenotype via dysregulation of PITX2. Clin Cancer Res 2017; 23: 298-310.

7) Andolfo I, Liguori L, De Antonellis P, Cusanelli E, Marinaro F, Pistollato F, Garzia L, De Vita G, Petrosino G, Accordi B, Migliorati R, Basso G, Iolascon A, Cinalli G, Zollo M. The micro-RNA 199b-5p regulatory circuit involves Hes1, CD15, and epigenetic modifications in medulloblastoma. Neuro Oncol 2012; 14: 596-612.

8) Yan C, Chen Y, Kong W, Fu L, Liu Y, Yao Q, Yuan Y. PVT1-derived miR-1207-5p promotes breast cancer cell growth by targeting STAT6. Cancer Sci 2017; 108: 868-876.

9) Bian EB, Chen EF, Xu YD, Yang ZH, Tang F, Ma CC, Wang HL, Zhao B. Exosomal lncRNA-ATB activates astrocytes that promote glioma cell invasion. Int J Oncol 2019; 54: 713-721.

10) Cui D, Sajan P, Shi J, Shen Y, Wang K, D… Zhou L, Hu P, Gao L. MiR-148a increases… cell migration and invasion by downre…ng GADD45A in human gliomas with IDH1 … mutations. Oncotarget 2017; 8: 25345-25361.

11) Xu Y, Xu W, Lu T, Dai Y, Liang W. MiR-126 affects the … vasion and migration of glioma cells t…gh GATA4. … Artif Cells Nanomed Biotechnol 20…

12) Zhang T, Ma G, Zhang Y, Huo … 599 inhibits proliferation and inva… glio… rgeting periostin. Biotechnol … 2017; 39: 1… 33.

13) Zhang Y, Wu Z, Li L, Xie … iR-30a inhibits … ma progression and stem … operties by … ression of Wnt5a. Oncol Rep … 1156 …

14) Xie X, Pan J, Han … Chen W. … tion of microRNA-532-… romotes the p… tion and invasion of bl… r can… ells throu…n promotion of HMG… Wnt/β… enin s…naling. Chem Biol Intera… 20… -81.

15) Yamada Y, Arai … Kojima … akamoto S, Komiya A, Na… Ichikaw… e of pre-miR-532 (miR-5… p and miR-5… regulation of gene expr… and molecular p… genesis in renal cell ca… J Clin Exp Urol 2019; 7: 11-30.

16) S… X, W… Y, Wang Y, Duan W. Loss of miR-532-5p i… omotes cell proliferation … metastasis by … encing CXCL2 expression … HCC. Am J Transl Res 2015; 7: 2254-2261.

Bender AM, Collier LS, Rodriguez FJ, Tieu C, Larson JD, Halde… Mahlum E, Kollmeyer TM, Akagi K, Sarkar G, …aespada DA, Jenkins RB. Sleeping …eauty-me… d somatic mutagenesis implicates …ormation of high-grade astrocyto…er Res 2010; 70: 3557-3565.

18) Komohara Y, Ohnishi K, Kuratsu J, Takeya M. Possible involvement of the M2 anti-inflammatory mac…e phenotype in growth of human gliomas. …ol 2008; 216: 15-24.

Komohara Y, Horlad H, Ohnishi K, Fujiwara Y, Bai B, Nakagawa T, Suzu S, Nakamura H, Kuratsu J, Takeya M. Importance of direct macrophage-tumor cell interaction on progression of human glioma. Cancer Sci 2012; 103: 2165-2172.