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Circular RNA circ_0067934 functions as an oncogene in glioma by targeting CSF1

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Abstract. – OBJECTIVE: The importance of circular RNAs in malignant tumors increases the attention of researchers. The role of circ_0067934 in glioma remains unclear. Our study aims to uncover how circ_0067934 functions in glioma development.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to determine the level of circ_0067934 in glioma tissues. Circ_0067934 was knocked down in glioma cells. Cell migrated and invaded ability was detected through functional assay *in vitro* and *in vivo*. Further mechanism assays were performed to explore the potential targets of circ_0067934.

RESULTS: The circ_0067934 was highly expressed in glioma tissues compared with adjacent samples. The expression of circ_0067934 was upregulated in glioma cell lines. The migrated and invaded ability of glioma cells was inhibited after circ_0067934 was knocked down. Besides, CSF1 expression decreased *via* knockdown of circ_0067934. Furthermore, tumor metastasis was inhibited after circ_0067934 was knocked down in nude mice.

CONCLUSIONS: The circ_0067934 could suppress cell migration and invasion of glioma by upregulating CSF1.

Key Words:
Circular RNA, circ_0067934, Glioma, CSF1.

Introduction

Glioma is the most common malignant tumor of the nervous system in the world, which brings a heavy burden to the public[1]. There are approximately newly diagnosed with glioma 100,000 people annually. Since the past decades, great progress has been made in the therapy of glioma. Glioma patients still suffer a deadly median survival which has the poorest five-year survival rate among all cancers[2,3]. Local recurrences or progression of distant metastasis contribute to the poor outcome. Furthermore, the occurrence and development of glioma are a multi-step, complex, and multi-stage process. Thus, it is extremely important to demonstrate new mechanisms underlying the development of glioma to find out potential therapeutic targets for human glioma.

With the development of high-throughput sequencing technology, circular RNAs (circRNAs) have been widely explored recently. Increasing evidence show that circRNAs play an important role in initiation and progression of several cancers. For example, hsa_circ_001988 is significantly down-regulated in colorectal cancer which is a novel potential biomarker and therapeutic target for colorectal cancer cases[4]. Serving as a sponge to miR-196a5p, circDOCK1 inhibits cell apoptosis in oral squamous cell carcinoma by targeting BIRC3[5]. Downregulating the expression of RhoA and circRNA_000839, miR-200b inhibits cell invasion and cell migration in hepatocellular carcinoma[6]. CircRNA 100146 functions as an oncogene and enhances cell proliferation and cell in non-small cell lung cancer through binding to miR-615-5p and miR-361-3p directly[7]. Recently, circ_0067934 is reported to be a novel oncogene in cancers. However, the function of circular RNA in glioma remains unclear. In our work, circ_0067934 was upregulated in glioma samples and cell lines. Moreover, circ_0067934 inhibited cell migration and invasion of glioma *in vitro*. Furthermore, circ_0067934 overexpression decreased tumor formation and upregulated CSF1 in nude mice.

Patients and Methods

Tissue Samples

A total of 55 glioma tissues and para-cancer tissues were obtained at the Jilin Beihua University Affiliated Hospital. The prognosis of patients

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was analyzed. No radiotherapy or chemotherapy was performed before the surgery. This investigation received the approval of the Ethics Committee of Jilin Beihua University Affiliated Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human glioma cell lines (U251, U87, T98, and U373) and a normal human astrocyte (1800) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

After glioma cells were cultured for 24 h on 6-well plates, cells were transfected with lentivirus targeting specifically targeting circ_0067934 (shRNA) and control (GenePharma; Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Those GFP-positive cells were chosen for the following experiments.

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

TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) was used to separate the total mRNA from tissues and cells. The synthesis of complementary deoxyribose nucleic acid was conducted through reverse transcription kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primer sequences used for RT-qPCR were as follows: circ_0067934 forward: 3'-TACAAGTTC-CCCAATCCT-5'; circ_0067934, reverse: 3'-CACAAATCCCATCT-CC-5'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5'-CCAAAATCAGATGGGG-CAA-TGG-3' and reverse 5'-TGATGGCAT-GG-GTG-ATTC-3'. The mRNA expression level was normalized to GAPDH.

Transwell Assay

To detect cell migration, 2 ×10^5 transfected cells in 100 µL serum-free DMEM were transformed to the top chamber of a 24-well 8-µm culture insert (Corning, Corning, NY, USA). 20% FBS-DMEM was added to the lower chamber of the culture insert. 24 h later, the inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. An inverted microscope (×20) was utilized for counting invaded cells in three random fields. For the migration assay, invasion ×10^5 transfected cells in 100 µL serum-free DMEM were transformed to top chamber of 24-well 8-µm culture inserts (Corning, Corning, NY, USA) coated with 50 µL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). 20% FBS-DMEM was added to the lower chamber of culture inserts. 24 h later, the inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. An inverted microscope (×20) was utilized for counting invaded cells in three random fields.

Western Blot Analysis

Proteins were extracted from cells by Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to extract the target proteins which were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). These membranes were incubated with antibodies rabbit anti-GAPDH and rabbit anti-CSF1 (Cell Signaling Technology, CST, Danvers, MA, USA) used in this study, as well as goat anti-rabbit secondary antibody (Cell Signaling Technology, CST, Danvers, MA, USA). Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of protein expression.

Xenograft Model

After circ_0067934 was knocked down in cells, cells were replanted into NOD/SCID mice (6 weeks old). Tumor volume was calculated every 5 days as the formula (volume= length × width^2 × 1/2). Tumors were extracted after 4 weeks. This investigation was approved by the Animal Ethics Committee of Jilin Beihua University Affiliated Hospital.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA). Graph PAD 5.0 (GraphPad Software, Inc., La Jolla, CA, USA)

helped to present these consequences. The difference between the two groups was compared by Kaplan-Meier method and Student's *t*-test. The statistical significance was defined as $p<0.05$.

Results

The Expression of Circ_0067934 in Glioma Tissues and Cells

RT-qPCR was used to detect circ_0067934 expression in 40 glioma patients' tissue samples and corresponding samples. As shown in Figure 1A, circ_0067934 was higher in tumor tissue samples than in corresponding samples. Moreover, as it is shown in Figure 1B, the circ_0067934 expression level was higher in glioma cells than in normal human astrocyte (1800).

Downregulation of Circ_0067934 Inhibited Cell Migration and Invasion in Glioma Cells

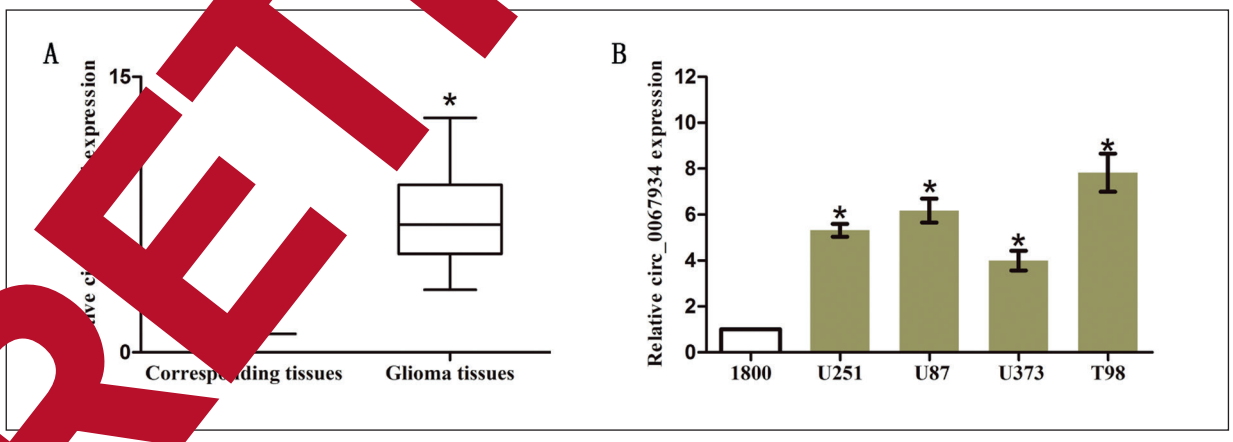
To explore the effect of circ_0067934 on glioma migration and invasion, wound healing assay and transwell assay were performed. T9 ll line was selected for transfection of circ_ lentivirus. RT-qPCR was used to meas he transection efficiency (Figure 2A). As sho Figure 2B, wound healing assay showed that migrated length of T98 cells wa educed aft transfection of circ_0067934 n Figure 2C the transwell assay sho that number of migrated T98 cells wa nifican educed after transfection of ci 7934 shown in Figure 2D, the number of invaded T98 cells was significantly reduced after t of circ_0067934 shRNA.

Downregulation of Circ_00 Repressed CSF1 in Gli a

Circular RNA Intera e (https://c actome.nia.nih.gov/) w sed to d the ta of circ_0067934. Re show at CSF1 was predicted as the t of _0067 and was reported t rti n num s cancers including oma. In wor e firstly researched nteraction b CSF1 and circ_0067 RT-qPCR w used to detect CS expre in T98 cells transfected with circ_0067934 or negative control (NC sults showed downregulation of c 0067934 decreased the mRNA expression CSF1 (Figur 3A). Moreover, the protein el of CSF1 measured through Western assay. The nregulation of circ_0067934 re the pr level of CSF1 (Figure 3B). RT-q sed to detect CSF1 expression in glioma tissues. Correlation analysis demonstrated that CSF1 expression level positively to circ_0067934 expression in glioma sue igure 3C).

Downregulation of Circ_0067934 Repressed Tumor Metastasis of Glioma In Vivo

To detect the ability of circ_0067934 *in vivo*, tumor metastasis assay was conducted in NOD/



ure 1. The expression level of circ_0067934 in glioma tissues and cell lines. **A,** Circ_0067934 expression was significantly ed in the glioma tissues compared with corresponding tissues. **B,** Expression levels of circ_0067934 relative to GAPDH w mined in the human glioma cell lines and normal human astrocyte (1800) by RT-qPCR. GAPDH was used as an inter ontrol. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

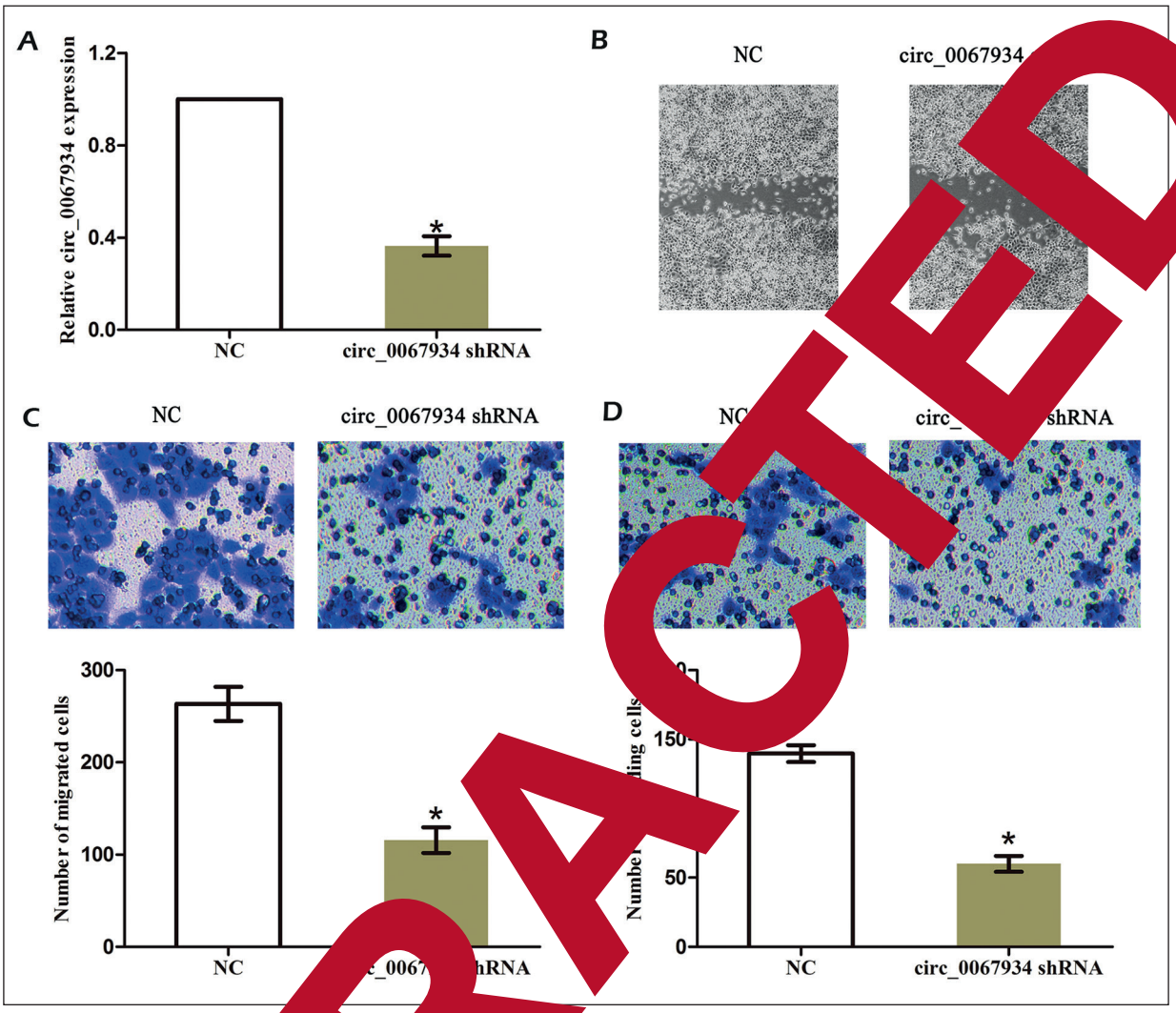


Figure 2. Downregulation of [illegible] inhibits glioma cell migration and invasion. **A,** Circ_0067934 expression in glioma cells transfected with [illegible] control (NC) or circ_0067934 shRNA (shRNA) was detected by RT-qPCR. GAPDH was used as an internal control. **B,** Wound healing assay showed that knockdown of circ_0067934 significantly repressed migrated [illegible] of glioma cells (magnification: 10×). **C,** Transwell assay showed that knockdown of circ_0067934 significantly repressed cell invasion in glioma cells (magnification: 40×). **D,** Transwell assay showed that knockdown of circ_0067934 significantly repressed cell invasion in glioma cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control group.

SCID [illegible] number of metastatic nodules in the lung [illegible] the circ_0067934 shRNA group significantly decreased compared to NC group (Figure 4A). After we extracted tumors from those treated mice four weeks later, circ_0067934 and CSF1 expression in those extracted tumor tissues were detected by RT-qPCR. As a result, circ_0067934 and CSF1 were lower-expressed in circ_0067934 shRNA group when compared with NC group (Figures 4B and 4C).

Discussion

Several researches have identified that circular RNAs are dysregulated in glioma and participate in the process of glioma development. For example, the occurrence and development of glioma is a multi-gene, multi-step, and multi-stage process. Furthermore, various tumor-specific molecular alterations have been associated with glioma, Hsa_circ_0001649 is downregulated in glioma

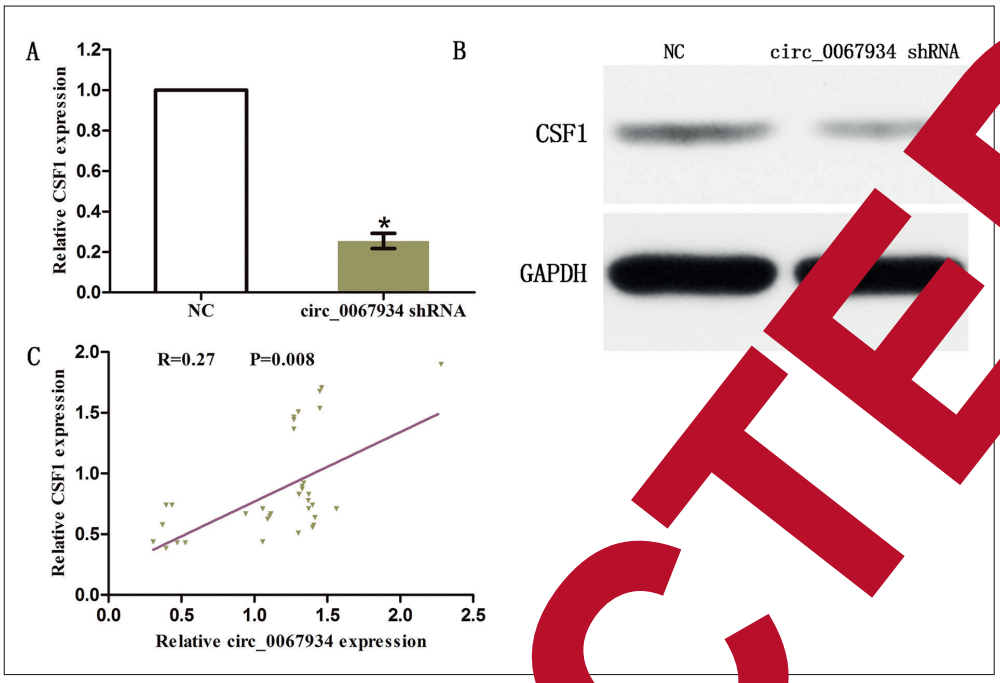
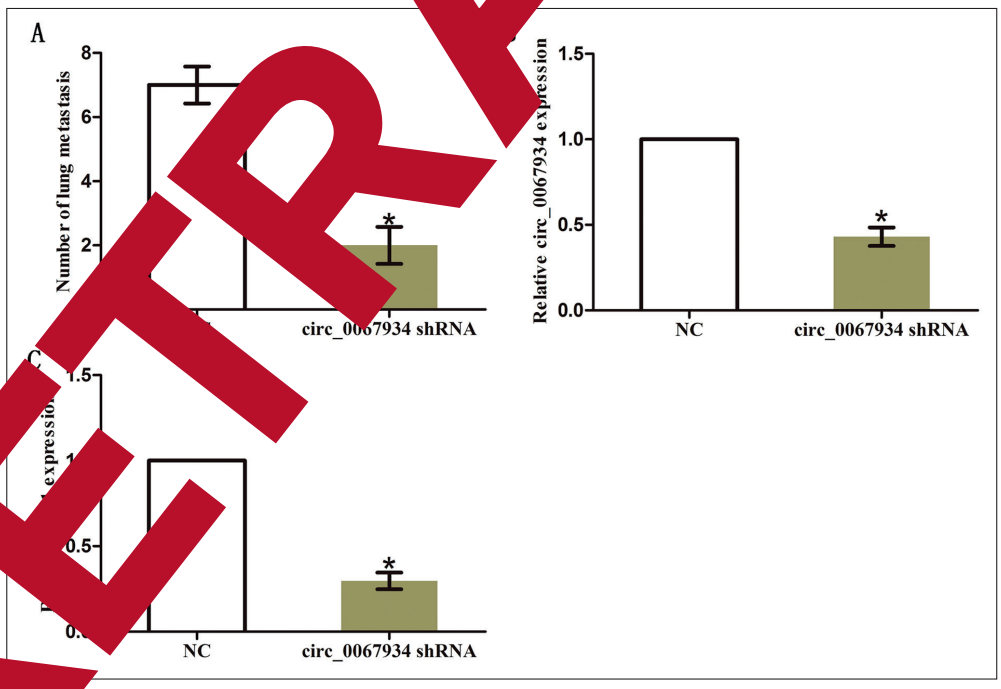


Figure 3. Downregulation of circ_0067934 inhibited CSF1 in glio... RT-qPCR ...ts showed that CSF1 expression was decreased in circ_0067934 shRNA group compared with NC group in ... Western blot results showed that CSF1 expression was decreased in circ_0067934 shRNA ...oup compared with ...up. **C,** The linear correlation between the expression level of CSF1 and circ_0067934 in Glio... *p<0.05.



...e 4. Dow...regulation of circ_0067934 inhibited tumor metastasis of glioma *in vivo*. **A,** The number of metastatic nodules ...e lung from the circ_0067934 shRNA group was significantly reduced compared to NC group. **B,** Circ_0067934 of those ...ed nodules was lower-expressed in circ_0067934 shRNA group compared with NC group. **C,** CSF1 of those dissected tu... circ_0067934 shRNA group and NC group. The results represent the average of three independent experiments. Data a...presented as the mean ± standard error of the mean. *p<0.05.

which predicts poor prognosis[8]. Through restraining the inhibition of miR-1236-3p on HOXB7 expression, hsa_circ_0074362 enhances cell proliferation, cell migration, and cell invasion[9]. Hsa-circ-0012129 facilitates proliferation and invasion of human glioma cells by targeting miR-661[10]. CircRNA TTBK2 functions as an oncogene in glioma *via* regulating miR-217/HNF1β/Derlin-1 pathway[11]. Our report showed that circ_0067934 was upregulated in glioma samples and cell lines.

Circ_0067934 is a circular RNA with a length of 170 nt which is generated from the chromosomal region 3q26.2. In recent years, circ_0067934 has been revealed a new target in various cancers. For instance, has_circ_0067934 is overexpressed in esophageal squamous cell carcinoma which promotes cell proliferation[12]. By regulating miR-1324/FZD5/Wnt/β-catenin pathway, circ_0067934 facilitates tumor growth and metastasis in hepatocellular carcinoma[13]. Circ_0067934 functions as an oncogene in the progression of cervical cancer by modulation of miR-545/EIF3C axis[14]. Circ-0067934 is upregulated in non-small cell lung cancer and promotes the cell proliferation of non-small cell lung cancer[15].

To determine the function of circ_0 in glioma metastasis, circ_0067934 R-NA was used for transfection in glioma Function assays showed that downregulation circ_0067934 repressed cell migration of glion cells. Moreover, we further ex effect o circ_0067934 on cell invas Res showed that downregulation of ci 067934 ributed to the decrement of inva gli these results indicated at 34 promoted cell migration an vasion ma.

The related p s of circ 34 were further explore Circular R nteractome (https: inte .nia.nih.gov/). Colony-stimulating factor 1(was found containing th nding area of 067934, which suggest CSF1 might be the arget protein of circ 934. is secreted by diverse cell typ hich ts its ffect through binding with ptor (C R). In the past years, CSF1 an 1R h been indicated to be ex- in s an cancers. For example, CSI SF1R b de improves the response of r reatic cancer cells to T-cell checkpoint im . Through modulating monodifferentiation and homing, CSF1 inhibits progression of breast cancer[17]. MiR-1207-5p i tumor growth and metastasis in lung canc *via* targeting CSF1[18]. Moreover, it has been reported that overexpression of CSF1 facilitates the progression of high-grade our report, results showed that d gulat of circ_0067934 decreased CSF pression *in vitro* and was positively corr with CSF1 expression in glioma tissues. I experiments *in vivo*, we found t down n of circ_0067934 decreased nor metast downregulated CSF1 i e mic The abo sults indicated that 0067 ight promote tumor metastasis o a th gh upr ating CSF1.

nclusions

We indicated that 0067934 was remarkabl lated in glio sues. Circ_0067934 p ted cell migration and invasion of glioma ugh upregulat g CSF1, which suggested that _0067934 m ontribute to therapy for glios a prospect target.

flict of Interest

declare that they have no conflict of interests.

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