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Circular RNA circ-SMAD7 promoted glioma cell proliferation and metastasis by upregulating PCNA

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Abstract. – OBJECTIVE: Recent studies have discovered a class of circular RNAs (circRNAs), which are dysregulated in various tumors and participate in the regulation of tumor progression. In our research, we aim to research the function of circ-SMAD7 in the progression of glioma.

PATIENTS AND METHODS: Circ-SMAD7 expression was detected by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) in glioma tissue patients. Pearson's Chi-square test was used to determine the associati of circ-SMAD7 expression with several pathological factors. Besides, cell prolif assay, cell cycle assay, transwell assay, d Matrigel assay were conducted to detect function of circ-SMAD7 in glioma. In additi the interaction between circ- and pr liferating cell nuclear antige glioma was studied by performin -PCR d Western blot assay.

RESULTS: Circ-SMAD served in glioma tis w pared with adjacent samples e exp n of circ-SMAD7 was as ted with p ts' WHO stage and KPS ell prolifera was inhibited and cycle regulated after circ-SMAD7 wa downregula glioma cells. Besides, c igration and ion were inhibited a circ-SMAD7 was wnregulated in glio ells. I dition, the mRNA and protei ressi of PCNA was repressed after circ- s kno down in glioma cells. Further PCNA pression level positive-relat ci MAD7 expression level in gli samp

C CLUSIO . Our study suggests that circ-SM tes proliferation and metastasis upregulating PCNA. Circ-SMAD7/PCNA might be a novel therapeutic strategy in ma.

Key Words:
Circular RNAs, Circ-SMAD7, Glioma, PCNA.

Introducti

Glioma mains f the most ordinary subtypes of malignant i nial cancers globally and e of the mo nal and aggressive t of cancers[1]. Glioma exerts heterogeneous acteristics ch brings a huge challenge e current t ments[2]. Despite therapeutic t ent devel for the last decades, the five-yea for patients remains the poorest ami cancers[3,4]. Therefore, the severe ituation underscores the urgency of figuring therapeutic interventions for the glioma ati

Circular RNAs (circRNAs) are characterized with evolutional conservation, enormous abundance and relative stability in cytoplasm. Recently, circRNAs play an important role in the initiation and progression of several cancers through sponging microRNAs (miRNAs) to regulate miRNAs' downstream genes or acting as competing endogenous RNAs (ceRNAs) for encoding RNAs. For example, the upregulation of hsa_circ_100395 significantly inhibits cell proliferation and reduces cell migration and invasion in lung cancer by targeting TCF21[5]. Circ_001988 is markedly down regulated in colorectal cancer which may be a novel potential biomarker and therapeutic target for colorectal cancer cases[6]. Circ_0067934 functions as an oncogene in cervical cancer by regulating the miR-545/EIF3C axis[7]. Through downregulating the expression of RhoA and circRNA_000839, miR-200b inhibits cell invasion and cell migration in hepatocellular carcinoma[8].Upregulation of circ-ITCH inhibits cell proliferation and cell metastasis in triple-negative breast cancer through regulating the Wnt/β-catenin pathway[9]. Recently, circ-SMAD7 is reported to be as a novel oncogene in cancers. However, how

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circ-SMAD7 functions in the proliferation and metastasis of glioma and the underlying mechanism remain unexplored.

In our work, circ-SMAD7 was remarkably upregulated in glioma tissues and cell lines. Circ-SMAD7 enhances cell proliferation and metastasis in glioma cell *in vitro*. Moreover, we further explored the underlying mechanism how circ-SMAD7 functioned in glioma development and found its function in tumorigenesis was associated with proliferating cell nuclear antigen (PCNA), which was reported to be an oncogene in many cancers including glioma.

Patients and Methods

Tissue Specimens

Paired tissues were sequentially enrolled from 46 glioma patients undergoing surgery in The Ninth People's Hospital of Suzhou from April 2016 to December 2018. This investigation was approved by the Ethics Committee of The Ninth People's Hospital of Suzhou. Signed written informed consents were obtained from all participants before the

Cell Culture

Human glioma cell lines (U87, U373, U and T98) and one normal human astrocyte 1 cell line were maintained in the e mediu consisted of 10% fetal bovine S; Thermo Fisher Scientific, Inc., nam, USA), penicillin as well as Dul 's Mod 's Medium (DMEM; The Waltham, MA, USA 37°C midified atmosphere with 5%.

Lentivirus Expressing Short-Hairpin RNA and Cell Transfection

Lenti expressing hairpin RNA (shRN irecte against circ-SMAD7 were provid Ge arma (Shanghai, China). The com nt DNA e ding circ-SMAD7 was amplifi inser into pcDNA3.1 (Gene- na, S hai ina), which were then for tra ction cells with Lipofectamine 300 Invitrogen, Carlsbad, CA, USA). The de- te SMAD7 expression level in these s was conducted using quantitative real-time ymerase chain reaction (qRT-PCR).

RNA Extraction and QRT-PCR

Total RNA from tissues and cells were separated by using TRIzol reagent (Invitrogen, Carlsbad, CA). Then, the total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs) through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Da na). Thermocycling conditions wer follow 30 s at 95°C, 5 s for 40 cycles a C, 35 s at 60°C. The $2^{-\Delta\Delta Ct}$ method was util calculating relative expression. The primer s es are as follows: circ-SMAD7 ard 5′- A GAGAAATCTATTGGA C-3′, circ-S reverse 5′-GGTTTC TCCG CTTT , β-actin, forward 5′- GA CGTC GAG-GCT-3′ and re CACTT TG-GAAATGC-3′ relati ressi as calculated by pe ing the $2^{-\Delta}$ d.

Western Blot Analysis

Total proteins we lected from cells *via* rad noprecipitati ssay (RIPA) buffer a hen quantified by using a protein assay inchoninic method; Beyotime, Shang- China). The get proteins were separated b dium do yl sulphate-polyacrylamide gel s (SDS-PAGE). Then, they were t d with antibodies after replaced to the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Then, the ti-β-actin (Cell Signaling Technology, CST, Danvers, MA, USA) and rabbit anti- PCNA (Cell Signaling Technology, CST, Danvers, MA, USA) were used for incubation of these membranes. The Pierce enhanced chemiluminescence (ECL) was utilized for visualizing Western blotting Substrate Immunoreactive bands (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation of these treated cells was monitored by CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, 5 mg/mL CCK-8 was added at each point (0, 24, 48, and 72 h). OD450 was measured using Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA) after the cells were incubated for 1 h.

Cell Cycle Assay

2×10^5/mL cells were diluted by RNase A in 75% ice-cold ethanol overnight. And these cells were stained with propidium iodide (PI; 50 mg/mL; MultiSciences Biotech Co., Ltd, Hangzhou, China) in the dark for 30 min at 4°C. Then, they were measured with flow cytometer (FACScan, BD Bioscience, Franklin Lakes, NJ, USA).

Transwell Assay and Matrigel Assay

After transfection, 1×10^5 cells in 200 µL serum-free DMEM were replanted in top chamber (Corning, Inc., Corning, NY, USA) with or without 50 µg Matrigel (BD, Franklin Lakes, NJ, USA). DMEM and FBS was added to the lower chamber. Next, they were cultured overnight in an incubator supplemented with 5% CO_2 at 37°C. The top surface of chambers was were treated by methanol for 30 min after wiped by cotton swab. Then they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

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 presenting these consequences. The difference between two groups were compared by Student's *t*-test. The statistically significance was defined as $p<0.05$.

Results

Circ-SMAD7 Expression Level in Glioma Tissues and Cells

The circ-SMAD7 expression was detected via qRT-PCR in 46 glioma patients' tissue samples and matched adjacent samples. As in Figure 1A, circ-SMAD7 was significantly up-

ulated in tumor tissue samples compared with adjacent tissues. As shown in Table I, the expression of circ-SMAD7 was associated with patients' WHO stage and KPS score. Moreover, SMAD7 level of glioma cells was higher than that of normal human astrocyte 1800 cell line (Figure 1B). The results suggested that circPSMC3 might be associated with glioma development.

Knockdown of Circ-SMAD7 Inhibited Cell Proliferation in Glioma Cells

We chose U251 cells for knockdown of circ-SMAD7. Then, qRT-PCR was utilized for detecting the circ-SMAD7 expression in treated cells (Figure 2A). To explore how circ-SMAD7 affected glioma proliferation, CCK8 assay was performed and results showed that after circ-SMAD7 was knocked down, the cell growth ability of U251 cells was significantly repressed (Figure 2B). Besides, the effect of circ-SMAD7 on glioma cell cycle was also researched. As was shown in Figure 2C, the percentage of G0/G1 cells increased and the percentage of S cells was reduced after knockdown of circ-SMAD7 in U251 cells.

Knockdown of Circ-SMAD7 Inhibited Cell Migration and Invasion in Glioma Cells

To explore how circ-SMAD7 affected glioma migration and invasion, transwell assay and Matrigel assay were performed. The results of transwell assay revealed that after circ-SMAD7 was knocked down, the migrated ability of glioma cells was sig-

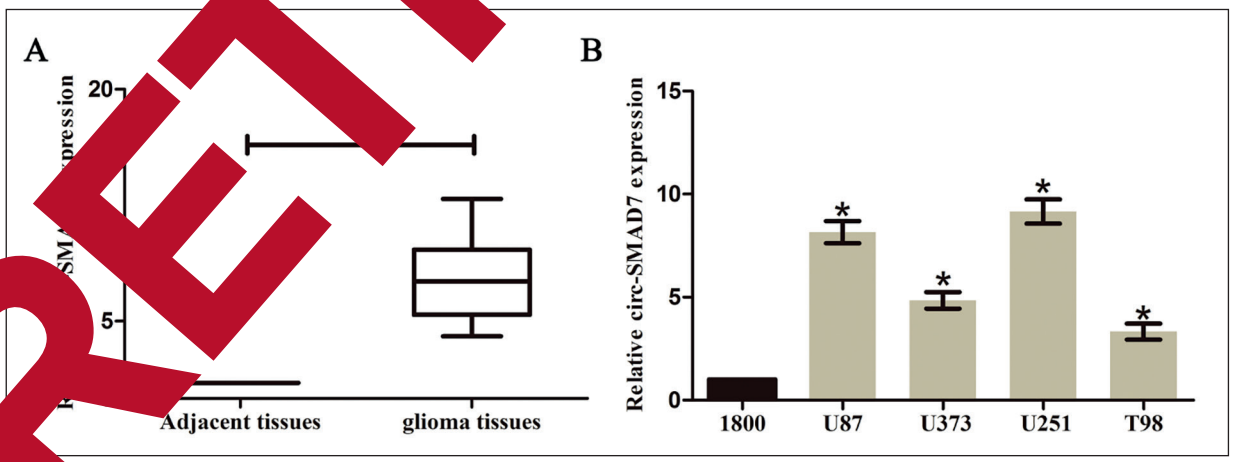

Figure 1. Expression levels of circ-SMAD7 were increased in glioma tissues and cell lines. **A,** QRT-PCR results showed that circ-SMAD7 expression was significantly increased in the glioma tissues compared with adjacent tissues. **B,** Expression levels of circ-SMAD7 relative to β-actin were determined in the human glioma cell lines and normal human astrocyte 1800 cell line by qRT-PCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

Table I. Correlation between circ-SMAD7 expression and clinicopathological characteristics in glioma patients.

Characteristics	Patients	Expression of circ-SMAD7		
		Low group	High group	
Total	46	20	26	
Age (years)				0.978
≤ 50	16	7	9	
> 50	30	13	17	
Gender				
Male	20	7	13	
Female	26	13		
WHO stage				0.014
II	25	15		
III-IV	21	5	16	
KPS score				0.0002
≥ 90	20	15	5	
< 90	26	5	21	

$p<0.05$ is considered statistically significant.

nificantly repressed (Figure 3A). In addition, Matrigel assay also revealed that after circ-SMAD7 was knocked down in glioma cells, the number of invaded cells was remarkably decreased (Figure 3B).

Th[illegible]action Betw[illegible] PCNA and C[illegible]SMAD7 in Glioma

qRT-PCR res[illegible] showed that expression lev- [illegible] PCNA in [illegible]ma cells was lower in circ-

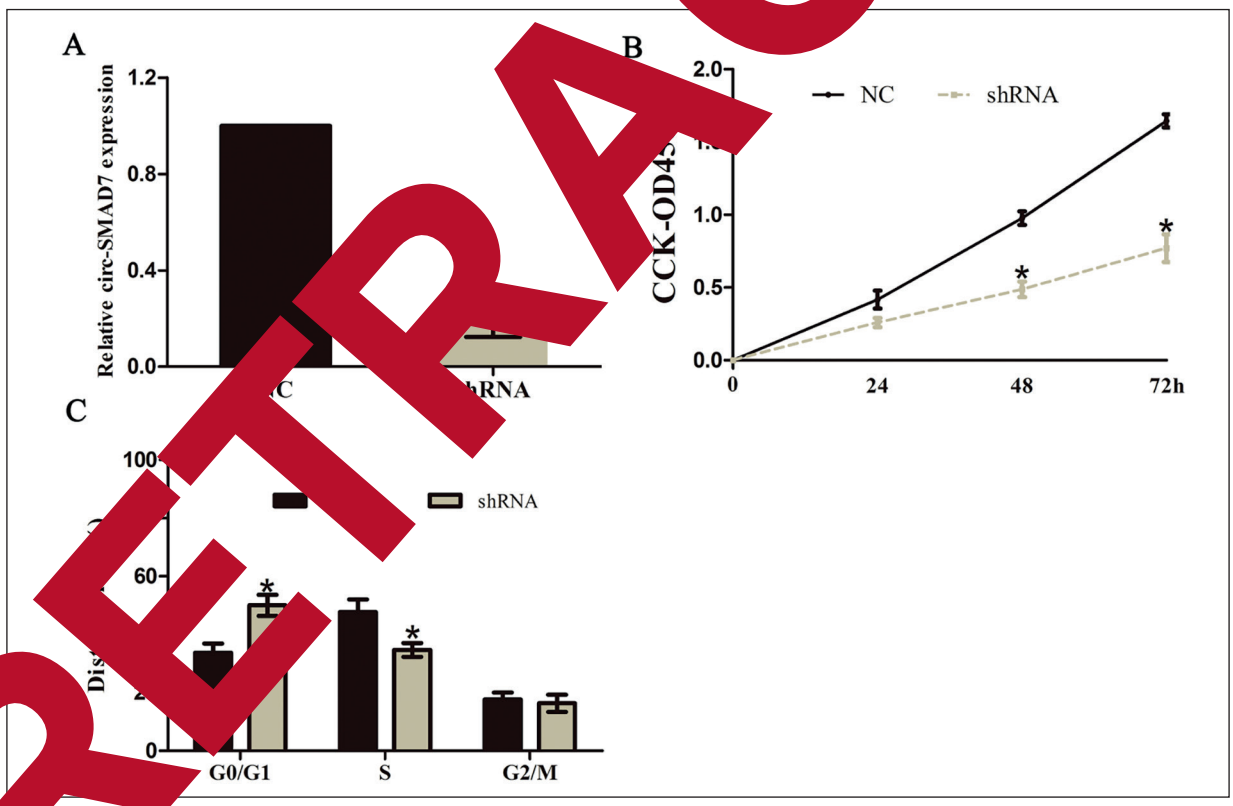

Figure 2. Knockdown of circ-SMAD7 inhibited glioma cell proliferation. **A,** Circ-SMAD7 expression in U251 glioma cells [illegible]ced with circ-SMAD7 shRNA (shRNA) and negative control (NC) was detected by qRT-PCR. β-actin was used as an in[illegible]ontrol. **B,** CCK-8 assay showed that knockdown of circ-SMAD7 significantly inhibited cell growth in glioma cells. **C,** Perce[illegible]tage of G0/G1 cells was increased and the percentage of S cells was reduced after knockdown of circ-SMAD7 in glioma cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

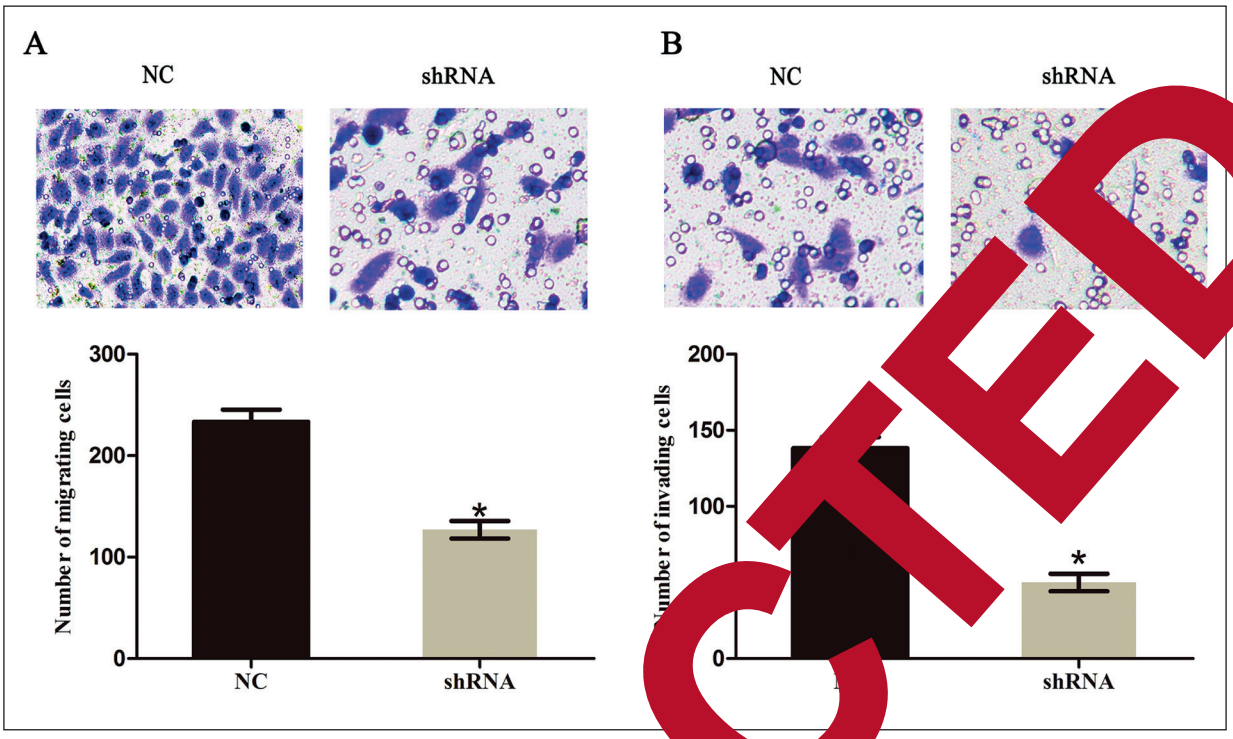

Figure 3. Knockdown of circ-SMAD7 inhibited glioma cell migration and invasion. **A,** Transwell assay showed that knockdown of circ-SMAD7 significantly decreased cell migration in glioma cells (magnification: 40×). **B,** Matrigel assay showed that number of invaded cells was significantly decreased after knockdown of circ-SMAD7 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 cells.

SMAD7 shRNA (shRNA) group decreased with the PCNA level in negative control (NC) group (Figure 4A). Western blot assay found that after circ-SMAD7 was knocked down, PCNA could be downregulated at protein level (Figure 4B). We further found that PCNA expression of glioma tissues was significantly higher compared with that of adjacent tissues (Figure 4C). Correlation analysis revealed that the positive association was seen between PCNA expression level and circ-SMAD7 expression in glioma tissues (Figure 4D).

Discussion

A number of researches has identified that circRNAs are dysregulated in glioma and participate in the process of tumor development. Increasing evidence has proved that circRNAs are potential indicators and therapeutic target for glioma. For instance, circ_0001649 is downregulated in glioma which predicts poor prognosis in glioma[10]. Circ-ITCH suppresses cells proliferation and induces cells apoptosis in epithelial glioma which is associated with prolonged overall survival[11]. Circ_0074362 enhances cell proliferation, cell migration, and cell invasion in glioma[12]. Circ_LARP4 is significantly down-regulated in glioma which may serves as a potential biomarker for prognosis of glioma patients[13]. CircRNA TTBK2 functions as an oncogene in glioma *via* regulating miR-217/HNF1β/Derlin-1 pathway[14]. Circ-001567 is upregulated in glioma and promotes cell proliferation and cell invasion[15].

CircRNA SMAD7, located in chromosomal 18, is reported to be overexpressed in esophageal squamous cell carcinoma and participate in regulating tumor development[16]. Our study showed that circ-SMAD7 was upregulated in glioma samples and cell lines. The expression of circ-SMAD7 was associated with patients' WHO stage and KPS score. After circ-SMAD7 was knocked down in glioma cells, glioma cell proliferation was found to be inhibited and cell cycle distribution was regulated. Moreover, glioma cell migration and invasion were also found to be inhibited after circ-SMAD7 was knocked

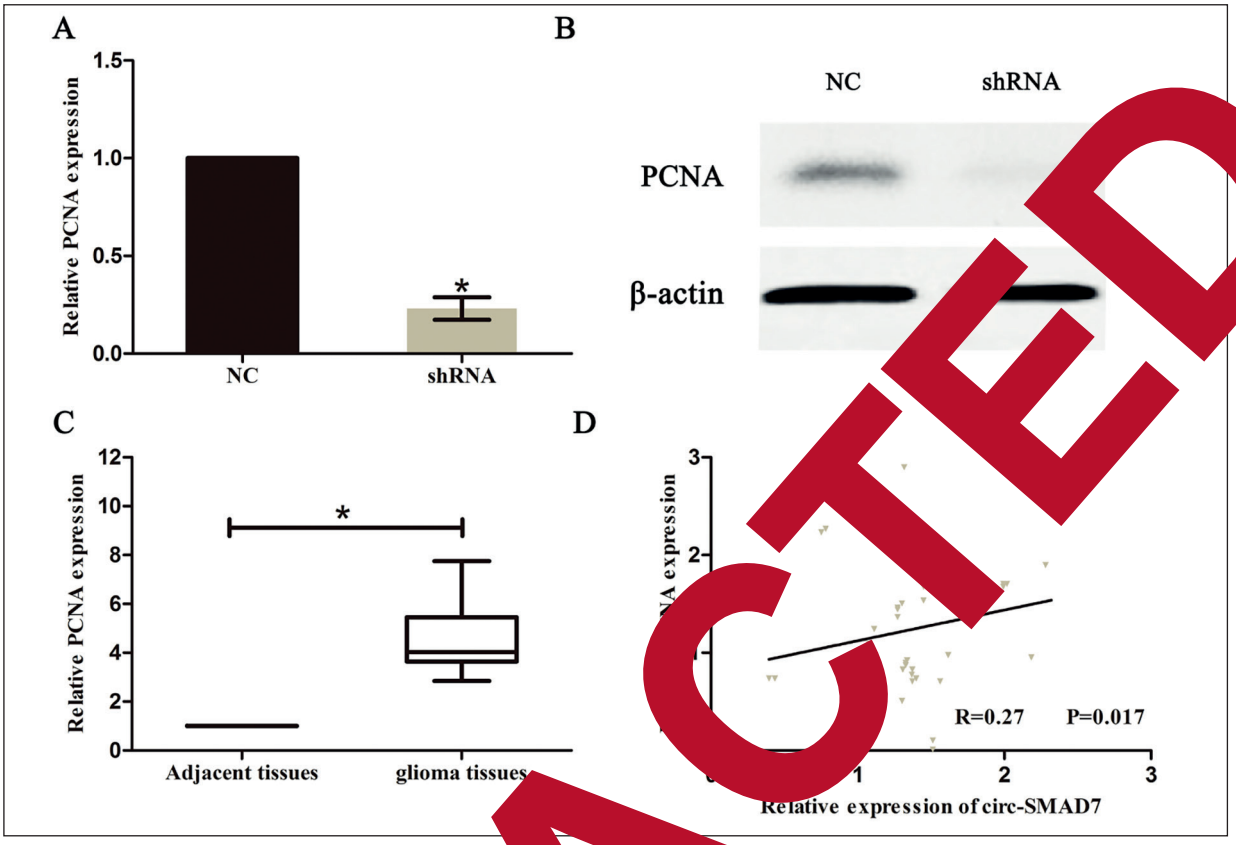

Figure 4. Interaction between circ-SMAD7 and PCNA in glioma cells. **A,** QRT-PCR results showed that PCNA expression was lower in circ-SMAD7 shRNA (shRNA) group compared with the NC group. β-actin was used as an internal control. **B,** Western blot assay revealed that PCNA protein expression was decreased in circ-SMAD7 shRNA (shRNA) group compared with the NC group. **C,** PCNA was significantly upregulated in glioma tissues compared with adjacent tissues. **D,** Linear correlation between the expression of PCNA and circ-SMAD7 in glioma tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05.

down. Above results indicated that circ-SMAD7 promoted tumorigenesis of glioma and might act as an oncogene.

Then, we further explored the potential target proteins of circ-SMAD7 using bio-informative methods and experiments. Results showed that the potential target protein, proliferating cell nuclear antigen (PCNA), was significantly upregulated in glioma tissue samples. Known as an oncogene, PCNA takes part in the regulation of various biological processes in many cancers. For example, upregulating PCNA is closely related to poor prognosis of patients with ovarian cancer[17]. The positive expression rates of PCNA were 73% in breast cancer tumor and could be utilized for evaluating the prognosis of breast cancer[18]. PCNA is significantly upregulated in colorectal cancer, especially in those with liver metastasis, and can help evaluating liver metastasis in patients with colorectal cancer[19]. In the present work, PCNA expression could be downregulated *via* knockdown of circ-SMAD7, while PCNA protein level could also be downregulated *via* knockdown of circ-SMAD7. Moreover, PCNA expression in glioma tissues was positively related with circ-SMAD7 expression. All the results above suggested that circ-SMAD7 might promote tumorigenesis of glioma *via* upregulating PCNA.

Conclusions

Circ-SMAD7 was remarkably higher-expressed in glioma tissues and cells. Besides, circ-SMAD7 could enhance glioma proliferation, migration, and invasion through targeting PCNA. These findings suggest that circ-SMAD7 may contribute to therapy for glioma as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Avgeropoulos NG, Batchelor TT. New treatment strategies for malignant gliomas. Oncologist 1999; 4: 209-224.
2) 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.
3) Penas-Prado M, Gilbert MR. Molecularly targeted therapies for malignant gliomas: advances and challenges. Expert Rev Anticancer Ther 2007; 7: 641-661.
4) Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 2004; 70: 217-228.
5) Chen D, Ma W, Ke Z, Xie F. CircRNA hsa_circ_100395 regulates miR-1228/TCF21 pathway to inhibit lung cancer progression. Cell Cycle 2018; 17: 2080-2090.
6) Wang X, Zhang Y, Huang L, Zhang J, Pan F, Li B, Yan Y, Jia B, Liu H, Li S, Zheng W. Decreas… pression of hsa_circ_001988 in colorecta… and its clinical significances. Int J Clin Exp … ol 2015; 8: 16020-16025.
7) Hu C, Wang Y, Li A, Zhang J, Xue F, Zhu L. C… expressed circ_0067934 acts as an oncogene… facilitate cervical cancer prog… a the miR… 545/EIF3C axis. J Cell Ph… 4: 9225-9232.
8) Wang BG, Li JS, Liu … Q. Mi… suppresses the inv… sio… n… patocellular carci… by … ulating RhoA and circRNA_0… 9. Tumo… l 2017; 39: 10104283177…
9) Wang ST, L… L… Wang YF, Xie F… Q, Wang R, Wei Q, …ng YH, …R, Feng XH. Circ-ITCH regulat… triple-negati… ast cancer progression … gh the Wnt/beta… in pathway. Neopla… 2019; 66: 232-239.
10) Wang Y, Sui X, Zhao H, Cong L, Li Y, Xin T, Guo M, Hao W. Decreased circular RNA hsa_circ_0001649 predicts unfavorable prognosis in glioma and exerts oncogenic properti… and in vivo. Gene 2018; 676: 117-12…
11) Luo L, Gao Y, Sun X. Circ-ITCH … elates with small tumor size, decreased FI… ge and prolonged overall survival, and it in… lls proliferation while promotes cell … apopto… ithelial ovarian cancer. Cance… mark 201… 505-513.
12) Duan X, Liu D, Wang … ien Z. C… ar RNA … circ_0074362 pro… glio… l proliferation, migration, and … uating … hibition of miR-12… p o… 7 expre… DNA Cell Biol 20… 7: 917-92…
13) Zou T, W… Gao Y, Liang … cular RNA_LARP… pressed and … rves as a potentia… marke… arian cancer prognosis. Eur Rev Med Pharmac… 2018; 22: 7178-7182.
14) … Liu X, Xue Y, … W, Ma J, Xi Z, Que Z, … Y. TTBK2 circular RNA promotes glioma malignancy by regulating miR-217/HNF1beta/Derlin-1 pathway. … ematol Oncol 2017; 10: 52.
… ao L, Zhong … ang L. Upregulation of circular RNA VPS13… s-circ-001567 promotes ovarian … iferation and invasion. Cancer Biothe… harm 2019; 34: 110-118.
16) Zhang Y, Wang Q, Zhu D, Rong J, Shi W, Cao X. … regulation of circ-SMAD7 inhibits tumor pro… ion and migration in esophageal squamous cell carcinoma. Biomed Pharmacother 2019; 111: 596-601.
17) Wang X, Wang D, Yuan N, Liu F, Wang F, Wang B, Zhou D. The prognostic value of PCNA expression in patients with osteosarcoma: a meta-analysis of 16 studies. Medicine (Baltimore) 2017; 96: e8254.
18) Guo JL, Gu SQ, Li Y, Zhang XY. Evaluation of clinical significance of endoglin expression during breast cancer and its correlation with ER and PCNA. Eur Rev Med Pharmacol Sci 2017; 21: 5402-5407.
19) Yue SQ, Yang YL, Dou KF, Li KZ. Expression of PCNA and CD44mRNA in colorectal cancer with venous invasion and its relationship to liver metastasis. World J Gastroenterol 2003; 9: 2863-2865.