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Circular RNA hsa_circ_0011946 promotes cell growth, migration, and invasion of oral squamous cell carcinoma by upregulating PCNA

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Abstract. – OBJECTIVE: The importance of circular RNAs in malignant tumors has been well concerned nowadays. Oral squamous ell carcinoma (OSCC) is diagnosed prevale the world. Our study aims to uncover the p tial functions of hsa_circ_0011946 in OSC velopment.

PATIENTS AND METHODS: Real-Time-qua titative Polymerase Chain Re RT-qPC was performed to determin e le of hsa_circ_0011946 in OSCC ti s and ll lines. Hsa_circ_0011946 was k ed do OSCC cells. Biological func ns in OSCC were iden d by p ing cell proliferation assay, y formati say, wound healing assay, well assay underlying mechanis of hs rc_001194 n regulating OSCC gression explored by RT-qPCR and tern blot assa

RES S: Hs circ_001 was highly expre in OS ssues compared with adjacent sam lt w so upregulated in OSCC cell lines. The k n of hs rc_0011946 inhibited cell th, tion, nvasion in OSCC. The expion o s reduced via knockdown of hs rc_001 Furthermore, the expression of P in tumor ssues was positively correlated to n of hsa_circ_0011946.

CONCL SIONS: Hsa_circ_0011946 could pote cell growth, migration, and invasion of OSby upregulating PCNA, which may offer a therapeutic intervention for OSCC patients.

Key Words:
Circular RNA, Hsa_circ_0011946, Oral squamous cell carcinoma, PCNA. Circular RNA Hsa_circ_0011946, Oral squamous cell carcinoma.

roduction

Ora the sixth most common cancer worldwide, which is more prevalent in develop- ountries[1,2]. Oral squamous cell carcinoma is the major subgroup of oral cancer, accounting for approximately 3% of all newly diagnosed clinical cancer cases[3]. Due to the invisible changes in the oral cavity, the early detection of OSCC is extremely important to improve the unsatisfied prognosis[4,5]. Despite development has been made in the treatment and diagnosis for OSCC, the prognosis of OSCC remains poor, with the overall 5-year survival rate below 50%[6,7]. Distant metastasis or local recurrences of OSCC contribute to the poor outcome. Thus, it is essential and urgent to find out important molecular markers involving in the development of OSCC and figure out new therapeutic treatments for OSCC patients.

As a novel class of noncoding RNAs, circular RNAs (circRNAs) are formed by the junction of the 3′end and 5′end. With the development of high-throughput sequencing technology, circRNAs are indicated to participate in the process of gene expressions. Moreover, increasing evidence has revealed that circRNAs function as important factors in tumorigenesis by sponging miRNAs. In particular, as a miR-1252 sponge, hsa_circ_0043256 inhibits cell proliferation and induces cell apoptosis in non-small cell lung cancer[8]. Circ-ABCB10 facilitates cell proliferation

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and tumorigenesis in breast cancer by sponging miR-1271[9]. By regulating miR-1324/FZD5/Wnt/β-catenin signaling, circ_0067934 facilitates tumor growth and cell migration in hepatocellular carcinoma[10]. By competing with microRNA-150-5p, circRNA ZNF609 enhances tumor growth and metastasis of nasopharyngeal carcinoma[11]. However, the role of hsa_circ_0011946 in OSCC has not been studied.

In our report, hsa_circ_0011946 was upregulated in OSCC samples and cell lines. Moreover, knockdown of hsa_circ_0011946 inhibited tumor proliferation, migration, and invasion of OSCC *in vitro*. PCNA has been reported to participate in the progression of many cancers including OSCC. Furthermore, hsa_circ_0011946 knockdown was able to downregulate PCNA *in vitro*.

Patients and Methods

Tissue Samples

A total of 58 OSCC tissues and para-cancer tissues were obtained at the Fourth Affiliated Hospital, Harbin Medical University. The pro of OSCC patients was analyzed. None of th rolled OSCC patients had preoperative radiot apy or chemotherapy. The research was appro by the Ethics Committee of th Affiliate Hospital, Harbin Medical U sity. e signed written informed consent e obta from all participants before the tud

Cell Culture

Human OS lines Tca TSCCA, CAL-27, SC , and rmal human oral keratinocyt HOK) we rchased from the Ameri Type Culture lection (ATCC, Ma as, VA SA). Cells were cultured in Du 's ified Eagle's Medium (DMEM; Hyclo th Lo UT, USA) supplement- ith fet ovine serum (FBS; Gibco, R ille, M A) in an incubator containing 5% O at 37 .

Cell Transfection

After OSCC cells were cultured for 24 h in l plates, the cells were transfected with lentivirus targeting specifically targeting hsa_circ_0011946 (shRNA) or control (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 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 utilized to separate the total mRNA from tissues and cells. The synthesis of c mentary deoxyribose nucleic acids (cD conducted through the reverse Tra ption Kit (TaKaRa Biotechnology Co., Ltd., China). The mRNA expression was no malize hat of glyceraldehyde 3-phosphate drogena AP-DH). QRT-PCR reaction ditions were lows: 94°C for 30 s, 55 r 30 s 72°C fo s, for a total of 40 cy es. ti pressio of the target gene was e sed b $^{-\Delta\Delta Ct}$ m . The primer sequen sed for R CR e as follows: Hsa 046, forwa GCTGGT-GTTCCTT CTG ; Hsa_cir 0011946, reverse: CACTGTAG ACCAGCATTTCT-5'; GA mers forward CAAAATCAGAT-G GCAATGCTGG-3′, reverse 5′-TGATGG-C GGACTGT TCATTCA-3′.

Cell Proliferation Assay

2× ted cells were seeded in 96-well plates. The cell proliferation was assessed by Cell feration Reagent Kit I (MTT; Roche, Basel, and) at 0 h, 24, 48, and 72 h following the manufacturer's protocol. Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Roche, Basel, Switzerland).

Colony Formation Assay

OSCC cells were inoculated in a 6-well plate for 10 days. Visible colonies were treated with 10% formaldehyde for 30 min and stained for 5 min with 0.5% crystal violet. The Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA) was used for data analysis.

Wound Healing Assay

OSCC cells were seeded in 6-well plates and incubated in DMEM overnight. Cells were scratched with a plastic tip and cultured in serum-free DMEM. Each assay was repeated in triplicate independently. The relative distance of wound healing was viewed under a light microscope (Olympus, Tokyo, Japan) at 48 h.

Transwell Assay

After transfection, 1×10^5 cells suspended in 200 µL of serum-free DMEM were applied in the top chamber (Corning, Corning, NY, USA)

pre-coated with or without 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 10% FBS was added to the lower chamber. After overnight culture, the top surface of chambers was treated by methanol for 30 min after wiping off unpenetrated cells using a cotton swab. Then, they were stained in crystal violet for 20 min. Five fields per sample were randomly chosen and captured under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

Western Blot Analysis

Protein was extracted from cells by the reagent radioimmunoprecipitation assay (RIPA; Yeasen, Shanghai, China). Dodecyl sulfate, sodium salt-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-PCNA (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, and goat anti-rabbit secondary antibody (Cell Signaling Techno… Danvers, MA, USA) at room temperatur… h. Image J software (NIH, Bethesda, MD, …) was applied for the assessment of the pr… expression.

Statistical Analysis

Data analysis was per…ed usin… tatistical Product and Service S…uti… CPS… Inc., Chicago, IL, …). Gra… 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) helped to present these consequences. The differences between the two groups were compared by the Student's *t*-test. The statistical significance was defined as $p<0.05$.

Results

The Expression of Hsa_ci… 001… in OSCC Tissues and Cells

RT-qPCR was used to d… t hsa_circ_00… expression in 58 OSC… ssues s… es and … jacent normal one… A… ow… n Figu… 1A, hsa_circ_0011946 express… as high… n tumor tissue sa… than that … r… samples. Moreover, h… 0011946 ex… n was also higher in O… CC ce… a8113, TS… CA, CAL-27, SCC-9… n that in … al human oral keratinoc… OK) (Figure … suggesting that the a… ant expression of hsa_circ_0011946 might b… sociated wi… OSCC development.

Do… egula… of Hsa_circ_0011946 Inhib… Proliferation in OSCC Cells

To explore the effect of hsa_circ_0011946 … OSCC proliferation, MTT assay, colony … n assay, and cell cycle assay were conducted. CAL-27 cell line was selected for transfection of hsa_circ_0011946 lentivirus. RT-qPCR was used to measure the transection efficiency (Figure 2A). MTT assay showed that the cell growth ability of OSCC was significantly repressed after hsa_circ_0011946 was

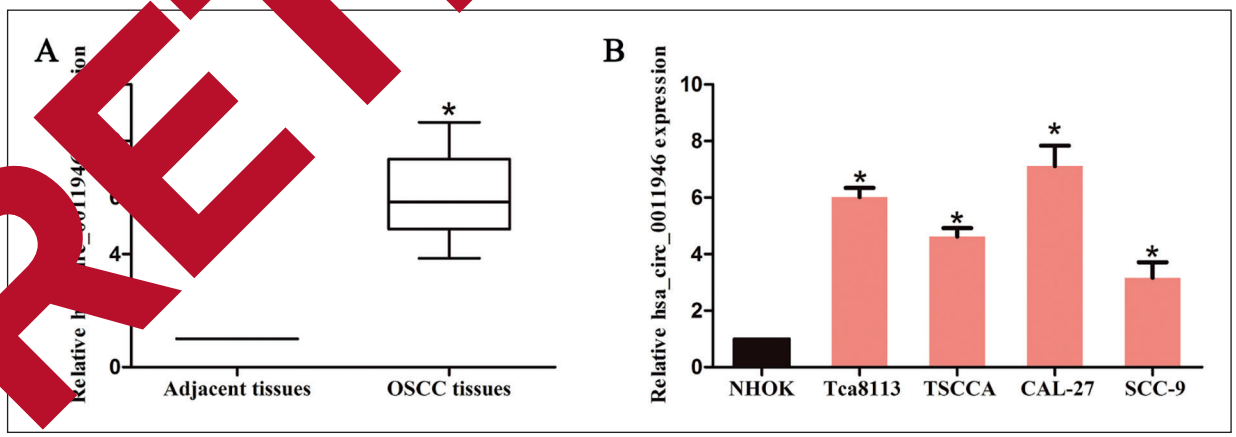

Figure 1. Expression levels of hsa_circ_0011946 were upregulated in OSCC tissues and cell lines. ***A,*** Hsa_circ_0011946 expression was significantly upregulated in the OSCC tissues compared with adjacent tissues. ***B,*** Expression level of hsa_circ_0011946 relative to GAPDH was determined in the human OSCC cell lines and normal human oral keratinocyte (NHOK) by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

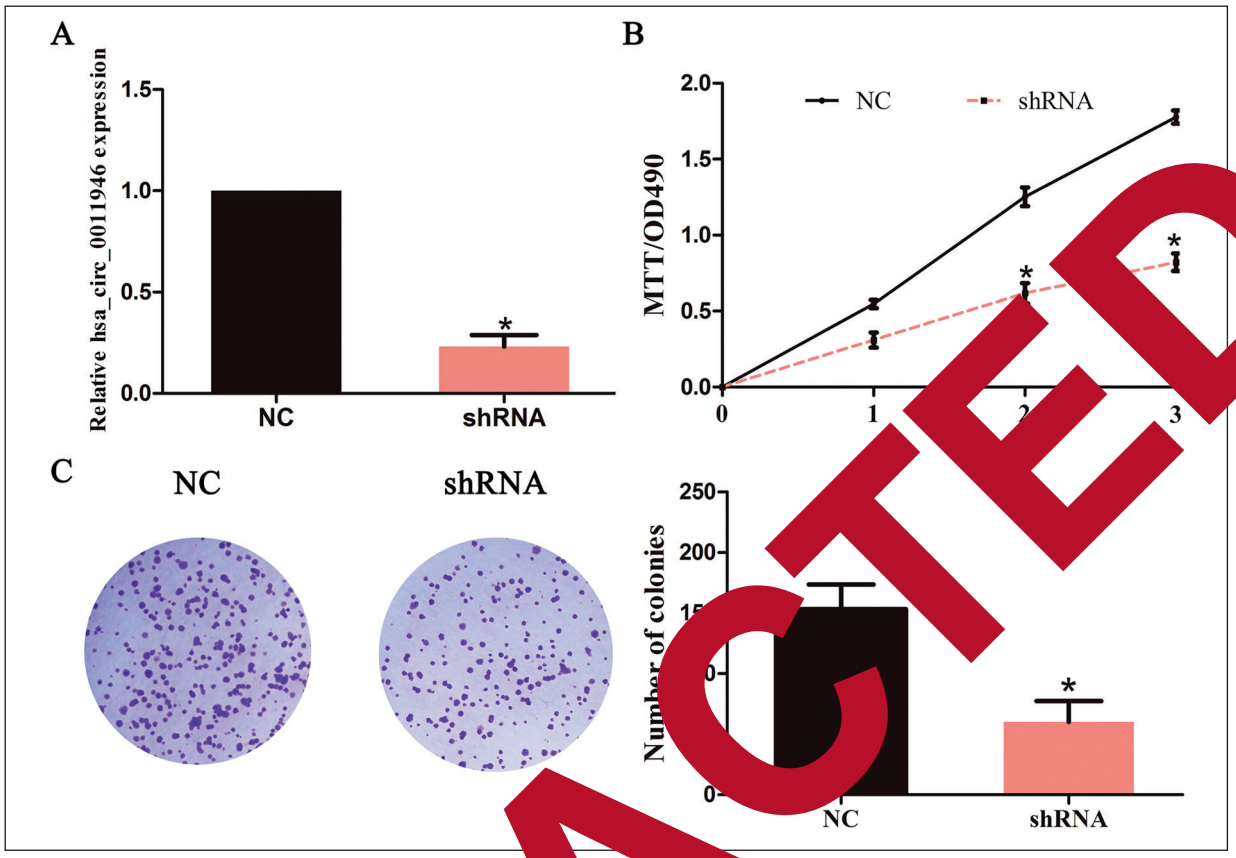

Figure 2. Downregulation of hsa_circ_0011946 inhibi[ted] S[CC] cell pr[olif]eration. ***A,*** Hsa_circ_0011946 expression in OSCC cells transfected with hsa_circ_0011946 sh[RN]A or NC w[as] [det]ected by RT-qPCR. ***B,*** MTT assay showed that downregulation of hsa_circ_0011946 significantly re[duced] viability [of] OSCC cells. ***C,*** Colony formation assay showed that the number of colonies significantly decreased [after] down[regu]lation of h[sa_c]irc_0011946 in OSCC cells (magnification: 40×). The results represent the average of three i[nde]pendent ex[peri]ments (me[an] ± standard error of the mean). *$p<0.05$, as compared with the control cells.

downregulated (Figure 2B). Colony formation assay showed that the colony number was significantly reduced after the knockdown of hsa_circ_0011946 (Figure 2C).

Downregulation of Hsa_circ_0011946 Inhibited Migration and Invasion in OSCC Cells

To explore the regulatory effects of hsa_circ_0011946 on migration and invasion of OSCC cells, wound healing assay and transwell assay were performed. As shown in Figure 3A, the migrated length of OSCC cells was reduced after the knockdown of hsa_circ_0011946. As shown in Figure 3B, the number of migratory cells remarkably decreased after the silence of hsa_circ_0011946 in OSCC cells. As shown in Figure 3C, after hsa_circ_0011946 was downregulated, the number of invasive cells was remarkably reduced.

Downregulation of Hsa_circ_0011946 Repressed PCNA Expression in OSCC

Our previous study showed that hsa_circ_0011946 could promote tumor proliferation and metastasis of OSCC. The downstream target of hsa_circ_0011946 remained unknown. To explore the potential targets of hsa_circ_0011946, Starbase v2.0 was utilized for the prediction. In our work, the interaction between PCNA and hsa_circ_0011946 was firstly researched. RT-qPCR was performed to detect PCNA expression in OSCC cells transfected with hsa_circ_0011946 shRNA or negative control (NC). Results revealed that the downregulation of hsa_circ_0011946 decreased the mRNA expression of PCNA (Figure 4A). Moreover, the protein level of PCNA was measured through the Western blot assay. Downregulation of hsa_circ_0011946 overexpression reduced the protein level of PCNA as well (Figure 4B). Besides, the Pearson correlation analysis

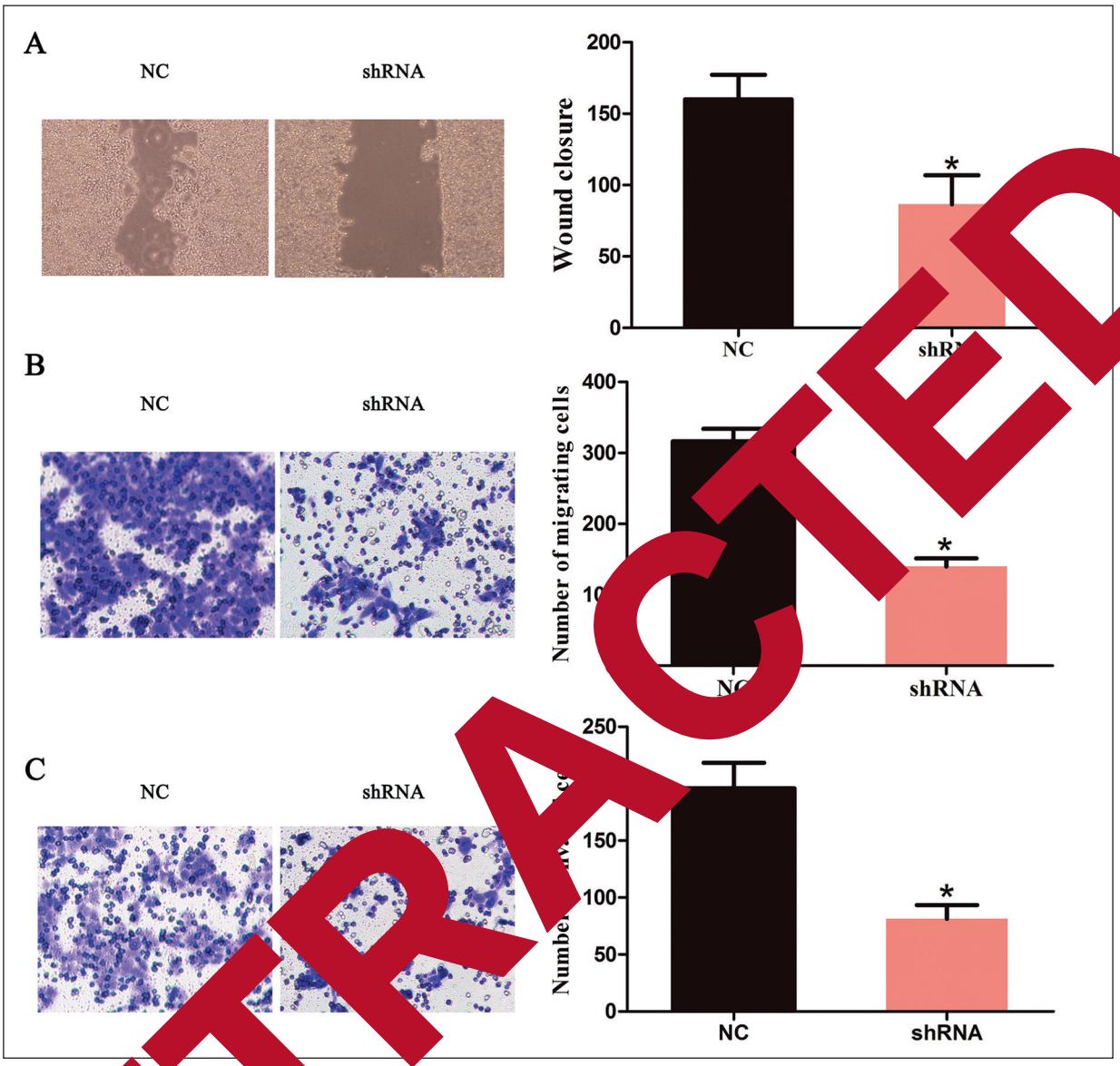

Figure 3 ...nregulation of ...c_0011946 inhibited OSCC cell migration and invasion. ***A***, Wound healing assay showed that kn...wn of hsa_circ_0011...gnificantly repressed migrated length of OSCC cells (magnification: 10×). ***B***, Transwell assa...wed tha...ckdown of hsa_circ_0011946 significantly repressed cell migration in OSCC cells (magnification: 40×). Trans...ssay showed that knockdown of hsa_circ_0011946 significantly repressed cell invasion in OSCC cells (magni...×). The...ts represent the average of three independent experiments (mean ± standard error of the mean). ...0.05, ...pared ...e control cells.

...that PCNA expression level was ...ositively correlated to hsa_circ_0011946 expres...n in OSCC tissues (Figure 4C).

Discussion

Increasing evidence has indicated the critical functions of circRNAs in modulating tumor development. Serving as a sponge to miR-196a5p, circDOCK1 inhibits cell apoptosis in OSCC by targeting BIRC3[12]. Downregulation of hsa_circ_0109291 suppresses cell proliferation and migration in OSCC, which may be a novel therapeutic target for OSCC[13]. By regulating the AKT/mTOR signaling, the upregulation of hsa_circ_0007059 restrains tumorigenesis and facilitates cell apoptosis in OSCC[14].

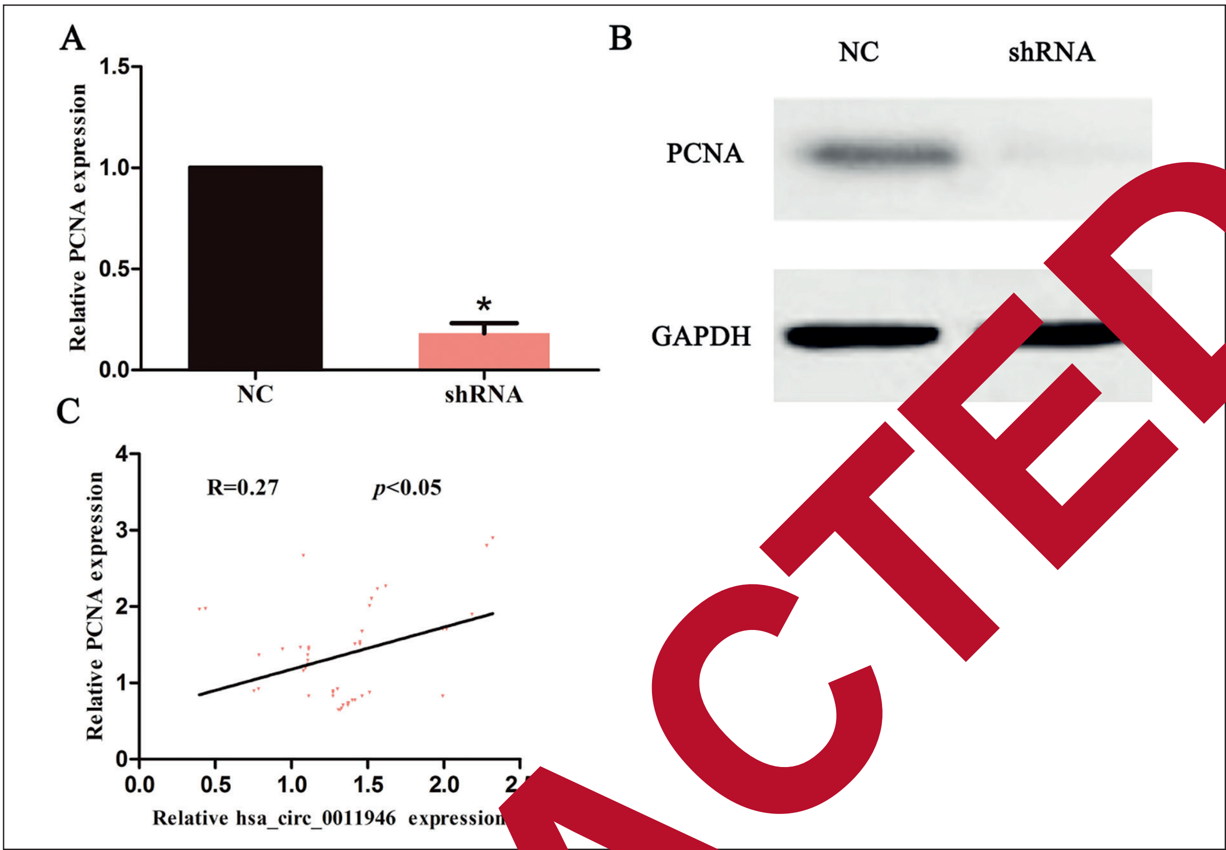

Figure 4. Downregulation of hsa_circ_0011946 inhib[illegible]CN[illegible] [illegible], RT-qPCR results showed that PCNA expression was downregulated in hsa_circ_0011946 shRNA grou[illegible]ed with NC group in OSCC cells. ***B,*** Western blot results showed that PCNA expression was d[illegible]ted in hs[illegible]_0011946 shRNA group compared with NC group. ***C,*** Linear correlation between the expression [illegible] and hsa_[illegible]0011946 in OSCC tissues. The results represent the average of three independent experiments. D[illegible]re pres[illegible]d as the m[illegible] standard error of the mean. $^*p<0.05$.

Through the EG[illegible]athway, o[illegible]pression of hsa_circ_0005[illegible]its cell [illegible]tion and proliferation [illegible] OSCC[illegible].

Hsa_ci[illegible]0011946 is a[illegible]l circRNA, which has be[illegible]eported to be do[illegible]gulated in many can[illegible]. In a[illegible]on, low expression of hsa_circ_[illegible]94[illegible]ibits cell migration and invasion in brea[illegible]er by t[illegible]ing RFC3[16].

[illegible] dete[illegible]e th[illegible]nction of hsa_circ_0011946 in [illegible]CC, [illegible]_0011946 shRNA was const[illegible]ed for t[illegible]sfection in OSCC cells. Func[illegible] showed that downregulation of [illegible]sa_circ_0011946 repressed proliferation of [illegible]SCC cells. We further explored the effect of [illegible]irc_0011946 on metastasis of OSCC cells. Re[illegible]ults showed that the downregulation of hsa_circ_0011946 contributed to the inhibition of migration and invasion in OSCC cells. All these results indicated that hsa_circ_0011946 inhibited tumor proliferation and metastasis of OSCC.

Then, the downstream proteins of hsa_circ_0011946 were further explored. Proliferating cell nuclear antigen (PCNA), initially discovered in cancer cells and proliferating cells, was predicted as the target of hsa_circ_0011946 through bio-informative analysis. Numerous reports have proved that PCNA is directly correlated to tumor differentiation, tumor staging, and prognosis. The expression level of PCNA is significantly higher in colorectal cancer, especially in those with liver metastases, which may help to assess organ metastatic condition in patients with colorectal cancer[17]. The positive expression rate of PCNA was 73% in breast cancer tissues, indicating the important clinical significance of PCNA in evaluating the prognosis of breast cancer[18]. Besides, the upregulation of PCNA is closely related to poor prognosis of patients with osteosarcoma, which may be a potential biomarker[19].

The potential interaction between PCNA and hsa_circ_0011946 was first researched in our study. Results showed that hsa_circ_0011946 knockdown decreased PCNA expression *in vitro* and was positively correlated with PCNA expression in OSCC tissues. Above results indicated that hsa_circ_0011946 might promote tumor development of OSCC by upregulating PCNA.

Conclusions

The above results provided evidence that hsa_circ_0011946 was remarkably upregulated in OSCC tissues, which promotes cell proliferation and metastasis of OSCC by upregulating PCNA. Our findings uncovered that hsa_circ_0011946 may contribute to therapy for OSCC as a prospective target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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