European Review for Medical and Pharmacological Sciences 2020; 24: 4665-4670

Caprin1 targeted by circular circ_0000885 in the tumor progression of osteosarcoma

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Abstract. – OBJECTIVE: Recent studies have revealed that circular RNAs (circRNAs) participate in the progression and development of many human diseases. Therefore, the purpose of this study was to uncover the role of circ_0000885 in the development of osteosarcoma and to explore the possible underlying mechanism.

PATIENTS AND METHODS: Circ_0000885 expression in osteosarcoma tissues and cells was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The knockdown of circ_0000885 was conducted in osteosa cells. Subsequently, its function in the p sion of osteosarcoma was determined ell proliferation assay, colony formation assay d cell cycle assay, respectively. Furthermore, underlying mechanism was explored by RT-q CR and Western blot.

RESULTS: Circ_0000885 hly expressed in osteosarcoma ues t that of adjacent tissues. Cell gr was s ficantly suppressed, and the d tion was regulated after irc_ lock-down *in vitro*. Besides, b the n and protein expressions of Ca were sig tly down-regulated *via* si of circ_0000 urthermore, Caprin1 itively corre ed with circ_000088 oste ma tissues.

CONCLUSIONS: Circ_ 5 could enhance osteosar cell prolifera d regulate cell cycle b egulating Caprin1, hich might contribut he th y for osteosarcoma.

Key

Circ irc_000 , Osteosarcoma, Caprin1.

Introduction

Osteosarcoma is one of the most common nary malignant bone tumors, especially in a nts, which often leads to death[1]. Osteosarc is characterized by a highly malignant tendency damage the s rounding tissues an etasta Despite improvements in treatment strategies e past 40 years, there has tle change i reatment strategies f etastatic osteosarcoma, and long-term survival rate of patie s has remained at 25%-30%[3-5]. Although recen vances in molecular biology provided in ts into the molecular pathoge of ost rcoma, the exact molecular mec steosarcoma remains unclear. Therefore, the study of novel and highly sensitive olecular biomarkers with reliable clinical significance is of great significance to improve the ogn s of osteosarcoma[6, 7].

Circular RNA (circRNA) is a kind of single-stranded endogenous closed cyclic RNA which does not have a 3'-terminal poly (A) and a 5'-terminal cap structure. CircRNA is a new type of RNA, which is different from traditional linear RNA. It has a closed ring structure and exists in a large number of eukaryotic transcriptomes. CircRNA is usually formed by splicing and cyclization of exons or introns of host genes. With the development of researches, circRNA has been found to be closely associated with the occurrence, development and prognosis of osteosarcoma, such as circ_HIPK3, circ_0001721, circRNA-0008717[8-10]. However, the role of circ_0000885 in osteosarcoma has not been explored. In this study, we aim to investigate the novel circRNA in osteosarcoma and the underlying mechanism.

Patients and Methods

Tissue Specimens

Totally, 44 paired osteosarcoma tissues and corresponding non-tumor tissues were collected from patients who underwent surgery at Cangzhou People's Hospital. Before surgery, none

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of these patients received radiotherapy or chemotherapy. All collected tissue samples were snap-frozen in liquid nitrogen immediately and stored at -80°C for the total RNA extraction. This study was approved by the Ethics Committee of Cangzhou People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human osteosarcoma cell lines (SOSP-9607, MG-63, and Saos-2) and osteoblastic cell line (hFOB 1.19) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37°C.

Cell Transfection

GenePharma provided us lentivirus expressing short-hairpin RNA (shRNA) against circ_0000885 (circ_0000885/shRNA) and negative control. Circ_0000885/shRNA was cloned into pGPH1/Neo vector (GenePharma, Shanghai, China) and transfected into MG-63 cells a[illegible] ing to the instructions of Lipofectamine 2[illegible] vitrogen, Carlsbad, CA, USA).

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

TRIzol reagent (Invitro[illegible] Ca[illegible]d, CA, USA) was used to extract t[illegible]otal RN[illegible] tissues and cells. Extracted RN[illegible] revers[illegible] into first-strand cDN[illegible] in [illegible]e with Reverse Transcription [illegible]em Kit [illegible]Ra, Dalian, China). Real Tim[illegible]antitative-P[illegible]T-qPCR) analyses utilize[illegible] Green I (Ta[illegible]Ka, Dalian, China) [illegible] we[illegible]formed in triplicate. The primer[illegible] used in this [illegible] were as follows: circ_000[illegible] primers fo[illegible] 5′-ACTGCCAGA[illegible]TGTCTCCC-3′, reverse: 5′-CGGGCCT[illegible]TTTC[illegible]CATC-3′; β-actin primers forwa[illegible]GAT[illegible]AAATC[illegible]TCAGAGGCT-3' and revers[illegible]GCAC[illegible]AGTTGGAAATGC-3'. The relat[illegible]ne e[illegible]ssion was calculated by $2^{-\Delta Ct}$ me[illegible] thermal cycle was as follow[illegible] sec at [illegible], 5 sec for 40 cycles at 95°C, and [illegible]c at 60°C.

Cell Viability Assay

A total of 2×10^3 cells was first plated into 96-w[illegible]tes. The Cell Counting Kit-8 (CCK-8, KeyGE[illegible]otech, Nanjing, China) was used to evaluate cell proliferation in accordance with the manufacturer's instructions. The absorbance v[illegible] ferent time point was determined b[illegible]icrop[illegible] reader, and the proliferation curve[illegible] plotted. Al[illegible] experiments were repeated for t[illegible]mes.

Colony Formation Ass[illegible]

For colony formation[illegible]y, specific [illegible] of transfected cells v[illegible] place[illegible] into six[illegible] plates. The cells we[illegible]ltured[illegible]complete medium containing 10[illegible]S f[illegible]o days[illegible]d the medium was re[illegible]ed e[illegible]ays. S[illegible]equently, formed col[illegible]s were fi[illegible]ith [illegible]anol and stained with[illegible] crystal vio[illegible]a-Aldrich, Saint Lou[illegible]SA) for 15 m[illegible]. The colony formatio[illegible]as de[illegible]d by counting the number of stained colonies.[illegible] wells were randomly sele[illegible] analysis for [illegible]reatment group.

Cell Cycle Assa[illegible]

RNase A so[illegible]n (250 μg/mL) was used to [illegible] the cells (2[illegible]) in 90% methanol solution f[illegible]minutes[illegible]°C. Then, the cells were incubate[illegible]ium iodide (PI) for 15 minutes. The Flow[illegible] software (Partec AG, Arlesheim, Switzerland) was finally used to detect cell cycle.

Western Blot Analysis

The cells were lysed in cell lysis buffer containing Tris-HCl and Triton X-100 to extract the total protein. An equal amount of denatured protein was separated by sodium dodecyl sulphate (SDS) polyacrylamide gel and transferred onto nitrocellulose membranes. After blocking with 5% non-fat milk in Tris-Buffered Saline and Tween-20 (TBST-20), the membranes were incubated with specific primary antibodies of anti-β-actin or rabbit anti-Caprin1 (Cell Signaling Technology, CST, Danvers, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 30 min. The protein bands were visualized using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA) and detected by ImageQuant LAS 4000 (Pittsburgh, PA, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis. GraphPad 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was applied for image editing. Student's *t*-test was used when appropriate. Quantitative data were

presented as mean ± SD (standard deviation). $p<0.05$ was considered statistically significant.

Results

Expression Level of Circ_0000885 in Osteosarcoma Tissues and Cells

In our study, RT-qPCR was conducted to detect circ_0000885 expression in 44 paired osteosarcoma tissues and 3 osteosarcoma cell lines. As a result, circ_0000885 expression was significantly upregulated in osteosarcoma tissues compared with adjacent non-tumor tissues (Figure 1A). Meanwhile, circ_0000885 expression in osteosarcoma cells was significantly higher than that of hFOB 1.19 (osteoblastic cell line) (Figure 1B).

Circ_0000885 Promoted Proliferation of Osteosarcoma Cells

MG-63 osteosarcoma cell line was chosen for the knockdown of circ_0000885 i*n vitro*. RT-qPCR was utilized to verify the transfection efficiency (Figure 2A). Subsequent the CCK-8 assay revealed that after circ_0000885 was kn[illegible]d down, the viability of MG-63 osteosarco[illegible] was significantly suppressed (Figure 2B). [illegible]y formation assay revealed that after circ_000[illegible] was knocked down, the number of colonies [illegible] remarkably reduced in MG-63 oste[illegible]arcoma cel[illegible] (Figure 2C).

Circ_0000885 Regula[illegible] Cell C[illegible] in Osteosarcoma Ce[illegible]

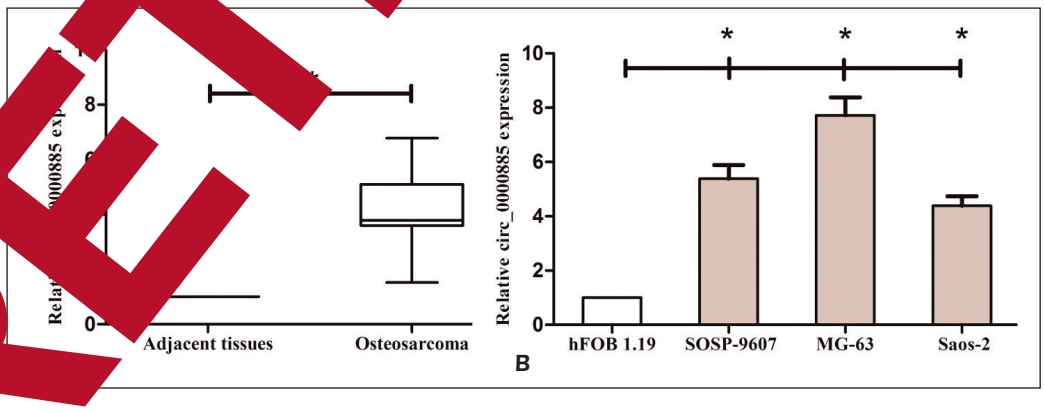
To identify the fu[illegible]tio[illegible]000885 in the cell cycle of os[illegible]arcoma [illegible]*ro*, we performed cell cycle assay. The results indicated that after circ_0000885 was knocked dow[illegible] osteosarcoma cells, the percentage [illegible]/G1 c[illegible] remarkably increased, while the [illegible]entage of S[illegible] cells decreased (Figure 3).

Interaction Between C[illegible]rin1 a[illegible] Circ_0000885 in Ost[illegible]arcoma

RT-qPCR results s[illegible]d that [illegible]e expre[illegible] level of Caprin1 w[illegible]emarka[illegible]lower in osteosarcoma cells o[illegible]000[illegible]/shRN[illegible]roup when compared [illegible]th t[illegible]ol gro[illegible](Figure 4A). Western [illegible] assay a[illegible]ow[illegible]hat after circ_000088[illegible]ockdown, Ca[illegible]was significantly do[illegible]d (Figure 4B). Furthermore, we dete[illegible]the C[illegible]1 expression in human tissues. The results del[illegible]ted that Caprin1 was high[illegible]ressed in ost[illegible]coma tissues com-p[illegible] with adjacent non-tumor tissues (Figure [illegible]. Besides, Ca[illegible]n1 expression level positively [illegible]related with [illegible]0000885 expression in os-[illegible]rcoma tissu[illegible](Figure 4D).

Discussion

[illegible] studies have shown that circRNA can [illegible]gula[illegible] gene expression by binding to DNA, RNA or protein at the transcriptional or post-transcriptional level in a homeopathic or trans-regulatory manner and participate in almost all physiological and pathobiological processes of human beings. Because of its structural closure and insensitivity to RNA enzymes it is more stable than linear RNA which are ideal tumor markers. It has shown that circRNAs have both positive and neg-



[illegible]re 1. Expression level of circ_0000885 increased significantly in osteosarcoma tissues and cell lines. **A**, Circ_0000885 [illegible]ion was significantly upregulated in osteosarcoma tissues compared with adjacent tissues. **B**, Expression level of ci[illegible]885 was detected in human osteosarcoma cell lines and hFOB 1.19 (osteoblastic cell line) by RT-qPCR. Data were prese[illegible]as mean ± standard error of the mean. *$p<0.05$.

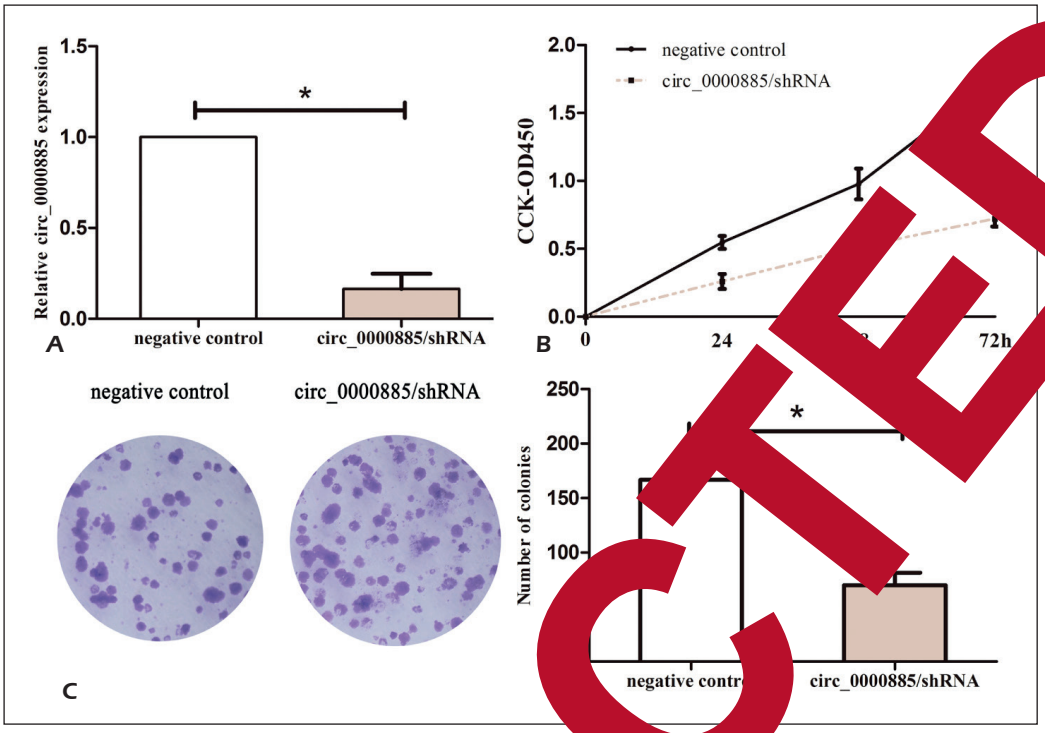


Figure 2. Circ_0000885 promoted osteosarcoma cell proliferation. **A**, 885 expression in MG-63 osteosarcoma cells transduced with circ_0000885 shRNA (circ_0 /shRNA) and negative control was detected by RT-qPCR. β-actin was used as an internal control. **B**, Cell prolifera that the viability of MG-63 osteosarcoma cells was significantly inhibited after the knockdown of circ_00 . **C**, on assay showed that the number of colonies was remarkably reduced via knockdown of circ_000088 G-63 a cells (magnification x 100). The results represented the average of three independent experiments (ard error of the mean). *p<0.05.

ative effects on the occurrenc lopment cancer, and induces lung metastasis of osteosarcoma in mice[18-20]. In addition, it can combine with other RBPs to influence the corresponding target genes, induce the formation of stress particles and

of tumors[11-15].

The early clinical sym s of os rcoma are relatively insidious. sult. are in the late stage ste at the time of diagnosis. Previ , circ 5 has been found to be sig ntly increas patients with osteosarc could serve a good diagnostic bi ker teosarcoma[16]. In this study, circ 0000885 was rkably upregulated in os rcoma tissues. ermore, after circ_00 85 wa knocked dow , cell proliferation signifi y inhibited. Meanwhile, after cir 0885 wnregu ion in osteosarcoma cells, cycle significantly regulated. The a result owed that circ_0000885 as a h gene in osteosarcoma.

Cycle A lated Protein 1 (Caprin-1) is a hi conserved cytoplasmic phosphorylated pr is located on chromosome 11 encodes 709 amino acids with a molecular ht of 116kDa[17]. Caprin-1 is a key regulator of p nscriptional regulation of gene. It can promote progression of gastric cancer and breast

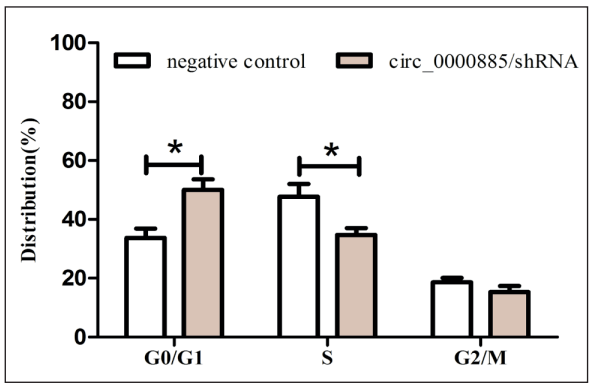


Figure 3. Circ_0000885 regulated osteosarcoma cell cycle. Cell cycle assay showed that the percentage of G0/G1 cells increased after the knockdown of circ_0000885 in MG-63 cells. The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

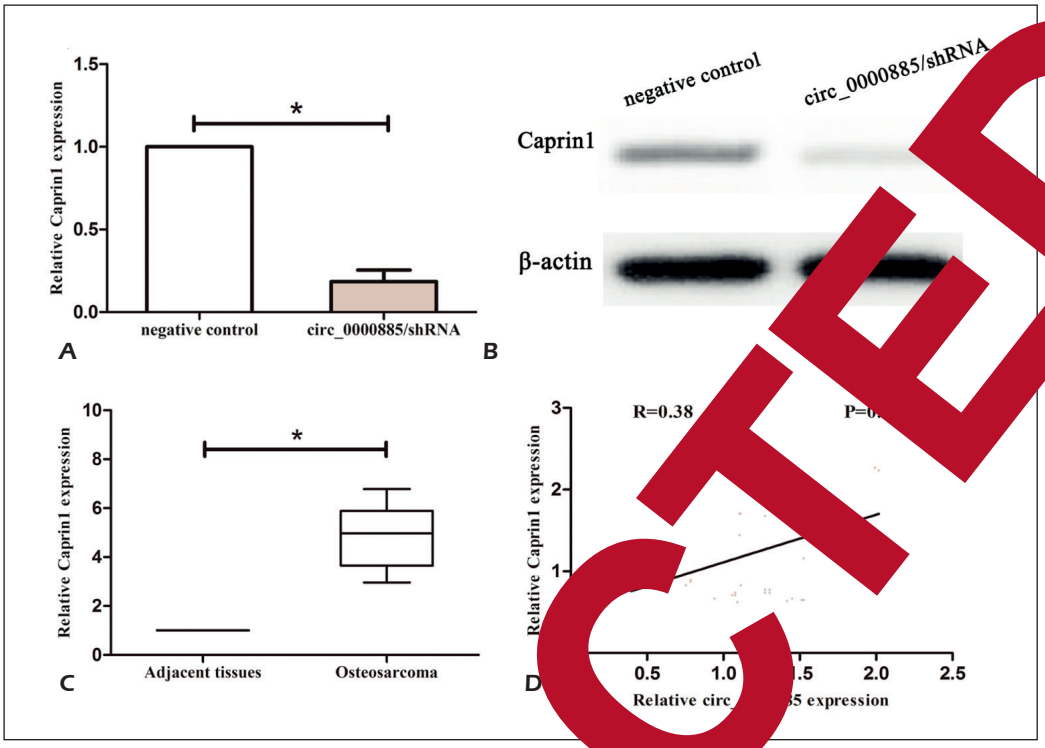


Figure 4. Interaction between Caprin1 and circ_0000885 in osteosarcoma. **A**, RT-qPCR results showed that Caprin1 expression was lower in circ_0000885/shRNA group with negative control group. **B**, Western blot assay revealed that Caprin1 protein expression decreased in circ_0000885/shRNA compared with the negative control group. **C**, Caprin1 was significantly upregulated in osteosarcoma tissues compared with adjacent tissues. **D**, The expression level of Caprin1 was positively associated with circ_0000885 in osteosarcoma tissues. The results represented the average of three independent experiments. Data were presented as mean ± standard error of mean. *p<0.05.

restrain the effects of cancer radiotherapy and chemotherapy.

In this study, 44 pairs of osteosarcoma and corresponding normal tissues were collected. Immunohistochemical staining showed that Caprin-1 was highly expressed in osteosarcoma. Therefore, we found that the expression level of Caprin-1 in osteosarcoma was positively correlated with the malignant degree of osteosarcoma. Caprin-1 is involved in the occurrence and development of glioma. Our results showed that Caprin1 expression was remarkably downregulated *via* knockdown of circ_0000885 in osteosarcoma cells. Furthermore, a positive correlation was observed between Caprin1 expression and circ_0000885 expression in osteosarcoma tissues.

Conclusions

We found that circ_0000885 was remarkably upregulated in osteosarcoma patients. Meanwhile, it could promote osteosarcoma cell proliferation and regulate cell cycle by upregulating Caprin1. Our findings provided a novel therapy target for osteosarcoma.

Conflict of Interests

The authors declared that they have no conflict of interests.

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