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LncRNA CCAT2 enhances cell proliferation via GSK3β/β-catenin signaling pathway in human osteosarcoma

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is a kind of malignant bone tumor. The aim of the manuscript is to investigate the clinical significance and the functional effects of long non-coding (lncRNA) colon cancer-associated transcript 2 (CCAT2) in osteosarcoma.

PATIENTS AND METHODS: Expression of CCAT2 was detected by quantitative Real-time PCR (qRT-PCR) in 50 cases of osteosarcoma tissue samples and adjacent normal bone tissues. Kaplan-Meier survival analysis and log-rank test were used to assess the association between CCAT2 expression and prognosis of OS patients. Cell Counting Kit 8 and cell colony formation assays were performed to evaluate cell proliferation. The protein expression of PCNA, p-GSK3β, GSK3β, and β-catenin were analyzed using Western blot analysis.

RESULTS: We demonstrated that lncRNA CCAT2 expression was significantly upregulated in OS tissues compared to adjacent normal bone tissues. Higher lncRNA CCAT2 expression positively associated with larger tumor size, advanced tumor stage and poor overall survival (OS) rate of patients. *In vitro*, knockdown of lncRNA CCAT2 suppressed cell proliferation and colony formation ability. In contrast, overexpression of lncRNA CCAT2 showed promoting cell proliferation effects in OS. Also, we found that knockdown of lncRNA CCAT2 inhibited GSK3β/β-catenin signaling by reducing p-GSK3β and β-catenin expression, but increasing GSK3β expression.

CONCLUSIONS: Our results showed that CCAT2 is a crucial oncogene in OS and may be a potential therapeutic target of OS.

Key Words:
Osteosarcoma, CCAT2, GSK3β, β-catenin, Cell proliferation.

Introduction

Osteosarcoma (OS) is identified as one of the major causes for cancer-related deaths in children and young adolescents[1]. In spite of chemotherapy largely improves the 5-year survival rate of OS patients, approximately 30-40% OS patients with localized osteosarcoma develop tumor recurrence or metastasis[2,3]. Systemic researches on molecular regulation mechanism are essential for investigating novel therapeutic strategies for OS patients.

LncRNA colon cancer-associated transcript 2 (CCAT2) is a novel lncRNA mapping to 8q24 genomic region. In the previous study, CCAT2 is identified as an oncogene in some tumor types[4]. For instance, long non-coding RNA CCAT2 promotes cell proliferation and invasion through regulating Wnt/β-catenin signaling pathway in clear cell renal cell carcinoma[5]. Upregulation of CCAT2 promotes cell proliferation by repressing the P15 in breast cancer[6]. High expression of CCAT2 indicates poor prognosis of gastric cancer and promotes cell proliferation and invasion[7]. However, the clinical significance and biological functions of CCAT2 in OS remain unknown.

In the study, we found that lncRNA CCAT2 expression was higher in OS patients. Higher lncRNA CCAT2 expression associated with prognosis of OS. *In vitro*, CCAT2 overexpression promoted cell proliferation and activated GSK3β/β-catenin signaling in OS. Thus, this evidence suggested that CCAT2 may be a crucial oncogene in OS and may be a potential therapeutic target of OS.

Patients and Methods

Clinical Tissue Samples

A total 50 cases of osteosarcoma tissues and adjacent normal bone tissues were obtained from November 2011 to January 2015 at Department of Orthopedics, the First Affiliated Hospital of Kunming Medical University. None of patients received any surgery operation, chemotherapy or

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radiation therapy before the surgery. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA analyses. All clinical procedures followed the protocols approved by the Ethical Committee of the First Affiliated Hospital of Kunming Medical University. Written informed consent was obtained from all patients. The clinicopathological factors of the patients were summarized in Table I.

Cell Lines Culture

The three human OS cell lines SAOS-2, MG63, and U2-OS and a normal osteoblast cell line (Nhost) were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells lines were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were supplemented with cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA).

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted by using TRIzol reagent (Qiagen, GmbH, Hilden, Germany). 1 μg of total RNA was reversed to complementary DNA (cDNA) by using Prime Script RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used to detect mRNA expression of CCAT2. The reaction was performed on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). GAPDH mRNA expression was normalized to expression of CCAT2. The mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ methods. The primer sequences were as follow: CCAT2-forward: 5'-CCCTGGTCAAATTGCTTAACCT-3', CCAT2-reverse: 5'-TTATTCGTCCCTCTGTTTTATGGAT-3'. GAPDH-forward: 5'-TGGTATCGTGGAAGGACTCAT-3', GAPDH-reverse: 5'-GTGGGTGTCGCTGTTGAAGTC-3'.

Cell Transfection

Two small interfering RNA targeting CCAT2 (si-CCAT2-1: sense: 5'-GCUAAGAGGAAACCACCUUTT-3', antisense: 5'-AAGGUGGUUUCCUCUUAGCTT-3', and si-CCAT2-2: sense: 5'-GCUCCACCUCUGACCAAUUTT-3'antisense: 5'-AAUUGGUCAGAGGUGGAGCTT-3') and negative control (si-NC) were generously provided by Thermo Fisher Scientific (Waltham, MA, USA). Human MG63 and U2-OS cell lines were transfected with si-NC, si-CCAT2-1, or si-CCAT2-2 using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

Cell Counting Kit 8 Assay

Human MG63 and U2-OS cell lines were used to assess cell proliferation ability using Cell Counting Kit-8 kit (CCK-8, Dojindo, Kumamoto, Japan) following the manufacturer's instruction. Briefly, cells (3000 cells/per well) were seeded in 96-well plates and transfected with si-NC or si-CCAT2. Cells were detected at the indicated time (0, 24, 48, and 72 hours) and CCK-8 solution was added to each well. After that, cells were maintained for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Cell proliferation was detected using a microplate reader victor (BioTek Instruments, Inc., Winooshi, VT, USA) and the absorbance was 450 nm.

Cell Colony Formation Assay

Human MG63 and U2-OS cell lines (500 cells/per well) were seeded at 12 well plates. Cells were transfected with si-NC or si-CCAT2. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 for 7 days. Transfected cells were fixed with 100% methanol, stained using 1% crystal violet and then cell colonies were counted.

Western Blot Assay

Human MG63 and U2-OS cell lines were lysed in radio immunoprecipitation assay (RIPA) buffer (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China). Protein samples were separated by 8-10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with specific antibodies including GSK3β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pGSK3β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Cell Signaling Technology, Danvers, MA, USA), followed by incubation with horseradish peroxidase (HRP)-linked secondary antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA). The protein bolts were detected using an enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All data analyses were carried out by SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The data results were showed as mean ± standard deviation (SD) and differences between groups were compared by Student *t*-test. $p<0.05$ was considered as statistical significance.

Results

CCAT2 Was Upregulated in OS Tissues and Correlated with Prognosis of Patients

To assess the expression pattern of CCAT2 in OS, we detected the expression of CCAT2 in 50-paired OS and adjacent normal bone tissues by qRT-PCR. The results showed that CCAT2 expression was upregulated in OS compared to adjacent normal bone tissues ($p<0.01$, Figure 1A). Moreover, we measured the expression of CCAT2 in three human OS cell lines including SAOS-2, MG63, and U2-OS cells and a normal osteoblast cell line (Nhost). Our results found that CCAT2 expression was upregulated in OS cells compared to Nhost cell ($p<0.01$, Figure 1B). According to median expression of CCAT2, we divided the patients into two groups (higher expression group and lower expression group). We analyzed the correlation between CCAT2 and clinicopathological factors of OS patients. The results showed that higher CCAT2 expression correlated with tumor size ($p=0.011$, Table I) and tumor stage ($p=0.042$, Table I) in OS patients. Survival plots by Kaplan-Meier analysis and log rank test demonstrated that higher CCAT2 expression predicted a poor overall survival of OS patients ($p<0.01$, Figure 1C).

CCAT2 Promotes Cell Proliferation and Cell Colony Formation of OS

Furthermore, we assessed the effects of CCAT2 expression on cell proliferation of OS. We performed gain-function assay in U2-OS cells and loss function assay in MG-63 cells, based on their CCAT2 expression in OS cells ($p<0.01$, Figure 1D). The si-CCAT2-2 was used to knockdown CCAT2 in U2-OS cells due to its higher knockdown efficiency. Results analyses of CCK8 cell proliferation assays showed that cell proliferation was inhibited by transfection of si-CCAT2 in U2-OS cells, but was enhanced by transfection of pcDNA3.1-CCAT2 in MG-63 cells, compared to their corresponding control groups ($p<0.01$, Figure 2A-2B). Results analyses of cell formation assays demonstrated that cell colony formed number was reduced by transfection of si-CCAT2 in U2-OS cells, but was increased by transfection of pcDNA3.1-CCAT2 in MG-63 cells, compared to their corresponding control groups ($p<0.01$,

Table I. Correlation of CCAT2 expression with clinicopathologic factors in 50 GC patients.

Clinicopathological factors	Total (n=50)	CCAT2 expression		*p*-value
		Lower (n=24)	Higher (n=26)	
Age (years)				0.749
≤ 55	22	10	12	
> 55	28	14	14	
Gender				0.706
Male	32	16	16	
Female	18	8	10	
Tumor size (cm)				0.011*
< 8	22	15	7	
≥ 8	28	9	19	
Lymph node metastasis				0.680
No	34	17	17	
Yes	16	7	9	
TNM stage				0.042*
IIA	28	17	11	
IIB/III	22	7	15	
Anatomic location				0.411
Tibia/femur	38	17	21	
Others	12	7	5	

*$p<0.05$.

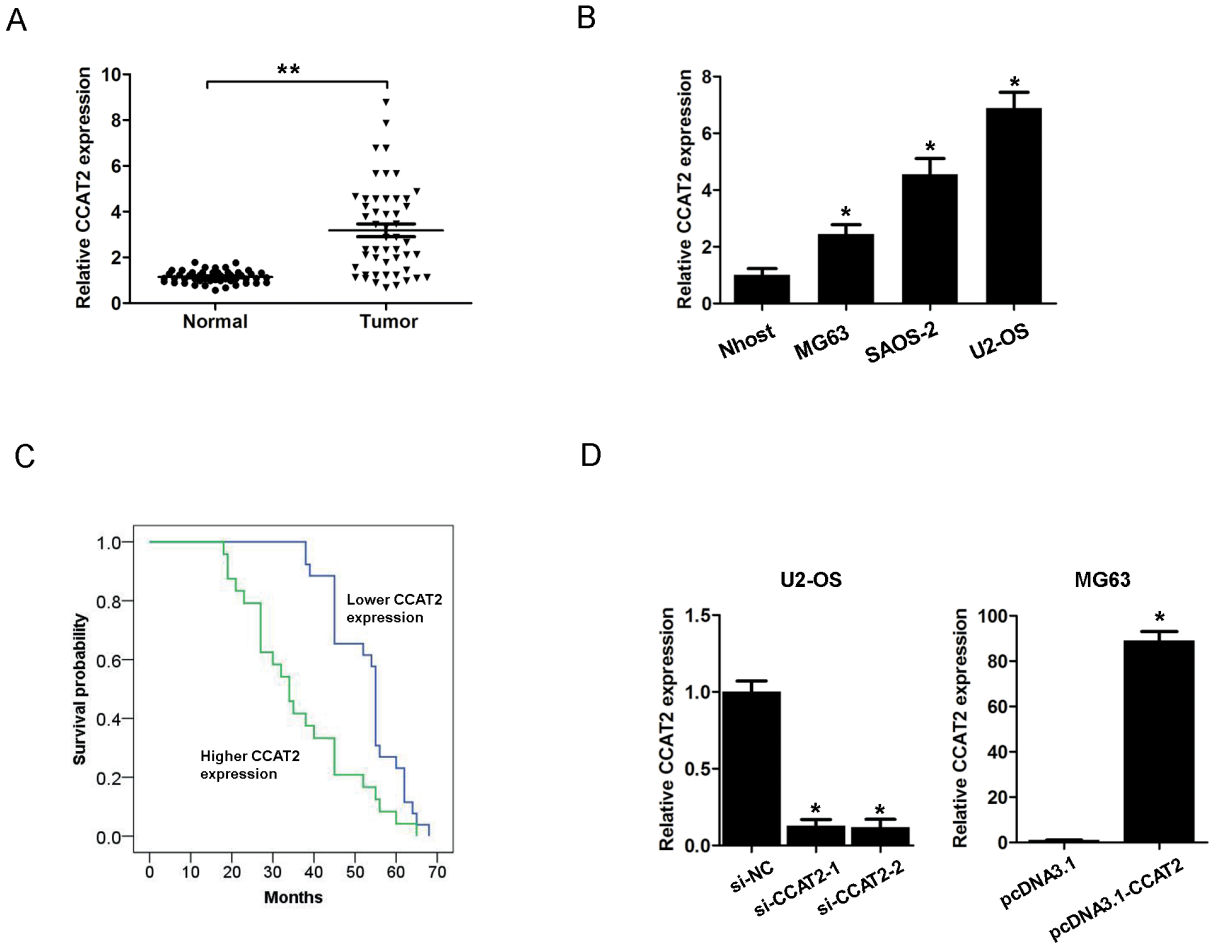

Figure 1. Expression of CCAT2 was significantly upregulated in OS tissues and cells. (A) Expression of CCAT2 was detected in 50 cases of OS tissues and adjacent normal bone tissues by qRT-PCR. (B) Expression of CCAT2 was detected in three human OS cell lines SAOS-2, MG-63, and U2-OS and a normal osteoblast cell line (Nhost) by qRT-PCR. (C) Survival plots by Kaplan-Meier analysis and log rank test demonstrated that higher CCAT2 expression predicted a poor overall survival of OS patients. (D) Expression of CCAT2 was detected after U2-OS cells were transfected with si-NC, si-CCAT2-1 and si-CCAT2-2 or MG-63 cells were transfected with pcDNA3.1-vector and pcDNA3.1-CCAT2 plasmids. Results were showed as mean ± standard deviation (SD), *p<0.05, **p<0.01.

Figure 2C-2D). Moreover, we demonstrated that cell proliferation related protein expression of PCNA was inhibited by transfection of si-CCAT2 in U2-OS cells. However, PCNA expression was increased by transfection of pcDNA3.1-CCAT2 in MG-63 cells compared to corresponding control groups (p<0.01, Figure 2E-2F). These results demonstrated that CCAT2 promoted cell proliferation in OS cells.

CCAT2 Promotes GSK3β/β-Catenin Signaling Pathway in OS Cells

The abnormal activation of GSK3β/β-catenin signaling pathway was significantly associated with tumor proliferation [8]. Our results demonstrated that knockdown of CCAT2 expression reduce the relative expression levels of p-GSK3β and β-catenin, but upregulating GSK3β expression in U2-OS cells (p<0.01, Figure 3A). However, upregulated CCAT2 expression increased the expression levels of pGSK3β and β-catenin expression, but downregulated GSK3β expression in MG-63 cells (p<0.01, Figure 3B). Thus, these results indicated that CCAT2 expression associated with pGSK3β/β-catenin signaling pathway in OS.

Discussion

The incidence of osteosarcoma in human is

not very high, but its high malignancy and strong invasion ability cause poor prognosis and high mortality of patients[9]. The 5-year survival rate of OS after amputation is less than 15%[10]. Thus, it is necessary to investigate new molecular targets for diagnosis and treatment of OS. LncRNAs have been identified as regulators of human tumors including osteosarcoma[11-13]. In the study, we found that CCAT2 expression was higher in OS tissues compared to adjacent normal bone tissues. Higher

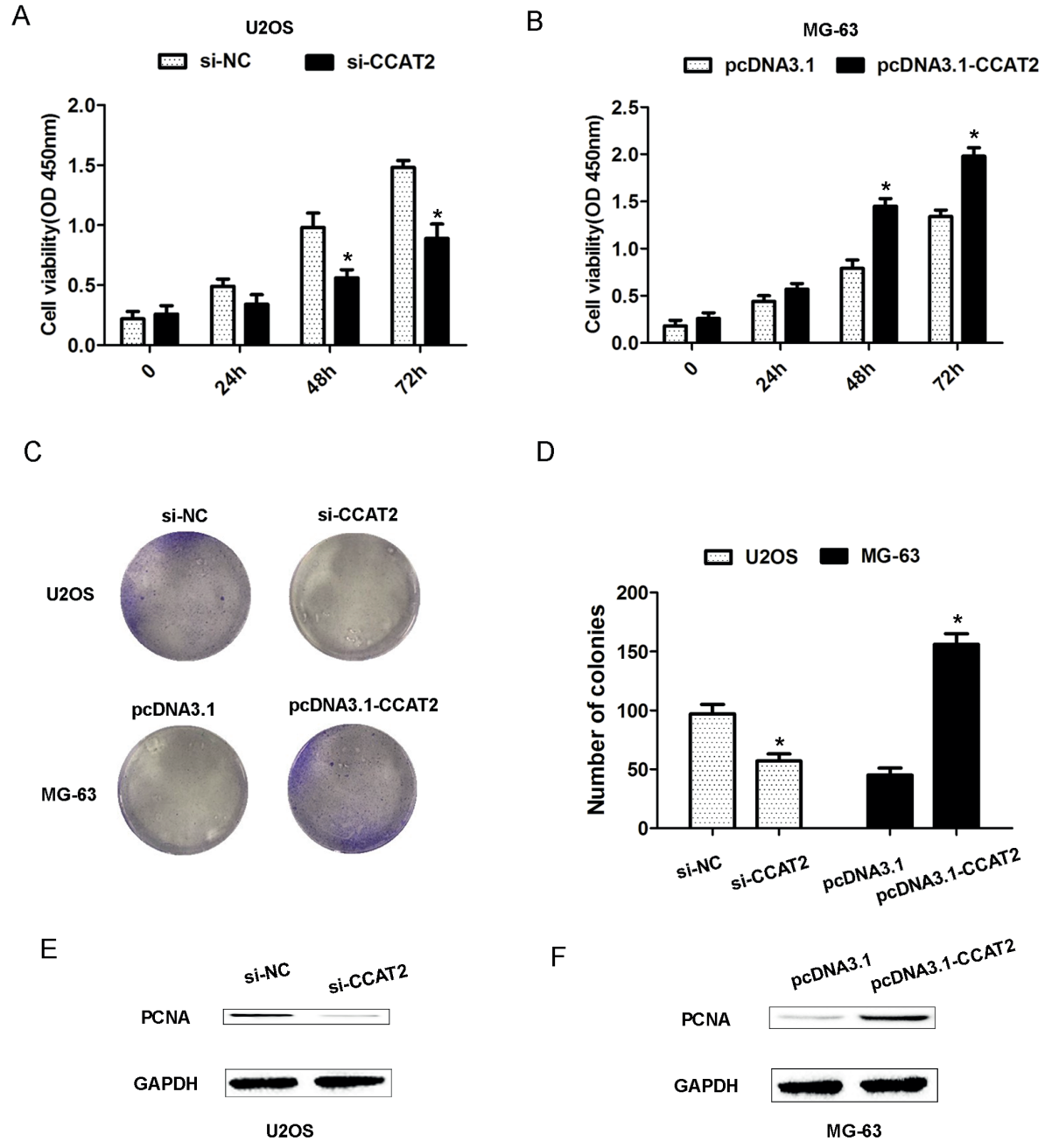

Figure 2. CCAT2 promoted cell proliferation *in vitro*. ***(A-B)*** CCK8 cell proliferation showed the cell proliferation ability after U2-OS cells were transfected with si-NC and si-CCAT2 or MG-63 cells were transfected with pcDNA3.1-vector and pcDNA3.1-CCAT2 plasmids. ***(C-D)*** Cell colony formation assay showed the cell colony formed number after U2-OS cells were transfected with si-NC and si-CCAT2 or MG-63 cells were transfected with pcDNA3.1-vector and pcDNA3.1-CCAT2 plasmids. ***(E-F)*** Expression of PCNA was detected using Western blot analyses after U2-OS cells were transfected with si-NC and si-CCAT2 or MG-63 cells were transfected with pcDNA3.1-vector and pcDNA3.1-CCAT2 plasmids. Results were showed as mean ± standard deviation (SD), * $p<0.05$.

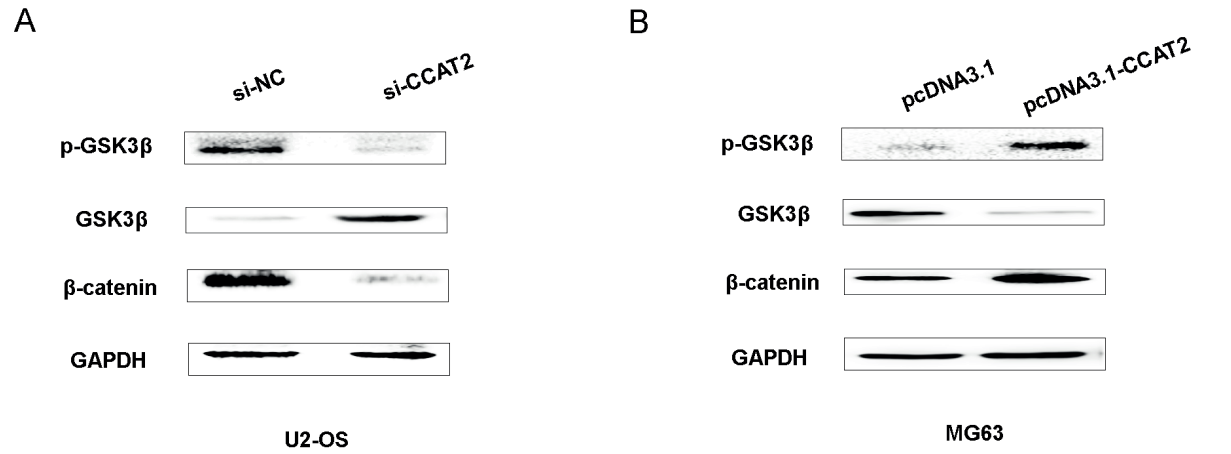

Figure 3. CCAT2 promotes GSK3β/β-catenin signaling pathway in OS cells. ***(A)*** Expression levels of p-GSK3β, GSK3β and β-catenin were detected using Western blot analyses after U2-OS cells were transfected with si-NC and si-CCAT2. ***(B)*** Expression levels of p-GSK3β, GSK3β and β-catenin were detected using Western blot analyses after MG-63 cells were transfected with pcDNA3.1-vector and pcDNA3.1-CCAT2 plasmids.

CCAT2 expression was associated with tumor size and TNM stage of OS patients. Moreover, the higher CCAT2 predicted a poor prognosis of OS. *In vitro*, we demonstrated that CCAT2 promoted cell proliferation and cell colony formation. However, knockdown of CCAT2 inhibited cell proliferation and cell colony formation ability. In previous study, CCAT2 was identified as oncogene in some tumors. Such as, knockdown of long noncoding RNA CCAT2 inhibits cellular proliferation, invasion, and epithelial-mesenchymal transition in glioma cells[14]. CCAT2 is associated with poor prognosis in hepatocellular carcinoma and promotes tumor metastasis by regulating Snail2-mediated epithelial-mesenchymal transition[15]. We showed that CCAT2 acted as oncogene in OS and promoted cell proliferation.

GSK3β/β-catenin signaling pathway was significantly associated with OS proliferation. In the previous study, CD151 knockdown inhibits osteosarcoma metastasis through the GSK-3β/β-catenin/MMP9 pathway[16]. Downregulation of tumor suppressing STF cDNA3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-3β/β-catenin/Snail signaling pathway. The overexpression of CARM1 promotes human osteosarcoma cell proliferation through the pGSK3β/β-catenin/cyclinD1 signaling pathway. We demonstrated that knockdown of CCAT2 expression reduce the relative expression levels of p-GSK3β and β-catenin, but upregulating GSK3β expression in OS cells. However, upregulated CCAT2 expression increased the expression levels of p-GSK3β and β-catenin expression, but downregulated GSK3β expression. Thus, these results indicated that CCAT2 expression associated with GSK3β/β-catenin signaling pathway in OS.

Conclusions

We showed that CCAT2 expression was upregulated in OS and associated with OS prognosis. *In vitro,* we found that CCAT2 promoted cell proliferation. Moreover, we demonstrated that CCAT2 expression was related to pGSK3β/β-catenin signaling pathway in OS. Thus, our results suggest that CCAT2 functioned as a potential target for patients with OS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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