

# Long non-coding RNA CCAT2 promotes prostate cancer cell proliferation and invasion by regulating the Wnt/ $\beta$ -catenin signaling pathway

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**Abstract.** Long non-coding RNA colon cancer associated transcript 2 (CCAT2) is dysregulated in a number of different types of human cancer, and affects cancer progression via the Wnt/ $\beta$ -catenin signaling pathway. However, the roles of CCAT2 and the Wnt/ $\beta$ -catenin signaling pathway in prostate cancer (PCa) are not completely understood. The present study aimed to investigate the potential mechanism of CCAT2 in PCa. In the present study, the reverse transcription-quantitative PCR (RT-qPCR) results indicated that CCAT2 expression was significantly upregulated in PCa tissues, and DU145 and PC3 cell lines compared with normal prostate tissues and the epithelial RWPE-1 cell line, respectively. Functional assays indicated that CCAT2 downregulation inhibited DU145 and PC3 cell proliferation, cell cycle, migration and invasion. In addition, the luciferase reporter assay, RT-qPCR and western blotting results indicated that CCAT2 regulated transcription factor 7 like 2 (TCF7L2) expression by binding to microRNA-217. Further western blotting and TOPFlash assays indicated that CCAT2-knockdown inhibited the Wnt/ $\beta$ -catenin signaling pathway in DU145 and PC3 cell lines by inhibiting the expression of TCF7L2. However, CCAT2-knockdown-mediated effects were reversed by the Wnt/ $\beta$ -catenin signaling pathway activator lithium chloride (LiCl). Further cell experiments suggested that LiCl treatment reversed CCAT2-knockdown-mediated inhibition of PCa cell proliferation, cell cycle, epithelial-mesenchymal transition, migration and invasion. Overall, the results indicated that CCAT2 regulated PCa via the Wnt/ $\beta$ -catenin signaling pathway; therefore, CCAT2 may exhibit key role during the progression of PCa and may serve as a therapeutic target for the disease.

## Introduction

Prostate cancer (PCa) is one of the most common malignant tumors in men worldwide (1). According to estimates in 2019 among the 10 leading types of cancer in the United States, PCa was the disease with the highest number of new cases in men (174,650/870,970 new cancer cases; 20%) and the second leading cause of cancer-associated death in men (31,620/321,670 deaths; 10%) (2). Despite significant advances in diagnostics, surgical techniques and adjuvant therapies over the past few decades, the morbidity and mortality rates of PCa have continued to rise rapidly (3). PCa is more prone to bone metastasis compared with cancer originating in any other tissue, which leads to excessive osteolysis and osteogenesis (4,5). Most patients with PCa inevitably develop castration-resistant PCa (CRPC) within two years, which is a more aggressive type of PCa (6). Bone metastases occur in the vast majority of patients with CRPC, resulting in less favorable prognosis and a significant increase in mortality; therefore, it is the most common cause of death in patients with PCa (7). A lack of effective treatment strategies is a major limitation for the treatment of recurrent and metastatic PCa (8). Therefore, improving the understanding of the molecular mechanisms underlying PCa progression is required for the effective treatment of patients with PCa.

Long non-coding RNAs (lncRNAs) are defined as transcripts >200 nucleotides in length with no apparent protein coding function (9). The role of lncRNAs in various biological processes, such as cell differentiation, proliferation and apoptosis in various diseases, including cancer, has received increasing attention (10-12). The colon cancer associated transcript 2 (CCAT2) gene has been reported to be upregulated in various types of human cancer, including gastric cancer, non-small cell lung cancer, esophageal cancer (13-17), hepatocellular carcinoma (18,19) and other tumors (20-22), and it is associated with the occurrence and metastasis of cancer, as well as poor prognosis. Previous studies have demonstrated that lncRNA CCAT2 is localized in the 8q24 genomic region, which has been identified as the first reproducible genetic risk site (23,24). However, to the best of our knowledge, the biological function and related molecular mechanisms underlying CCAT2 in PCa have not been previously studied.

Wnt is a cysteine-rich glycoprotein that is secreted by cells into the extracellular matrix (25). Activation of the

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Wnt/ $\beta$ -catenin signaling pathway alters receptor-mediated signaling, primarily by inhibiting the degradation of  $\beta$ -catenin in cells, which causes cytoplasmic  $\beta$ -catenin to accumulate and transfer to the nucleus, thereby affecting the transcriptional activity of the target gene (26,27). Alterations to the activity of the Wnt/ $\beta$ -catenin signaling pathway are often associated with inflammatory responses, cell proliferation and differentiation, transcriptional activity and cell membrane structure, which in turn affect the progression of various types of cancer, neuronal diseases, bone diseases and developmental disorders (28-30). An increasing number of studies have reported that abnormal Wnt/ $\beta$ -catenin signaling is associated with the development and progression of a variety of malignancies (31,32).

It has also been reported that by activating the Wnt/ $\beta$ -catenin signaling pathway, lncRNA CCAT2 affects oral squamous cell carcinoma, ovarian cancer and renal cell carcinoma (33), as well as non-small cell lung cancer, breast cancer, esophageal cancer and glioma (15,17,33-37). However, to the best of our knowledge, no studies have previously examined the relationship between CCAT2 and the Wnt/ $\beta$ -catenin signaling pathway in PCa, and its potential regulatory mechanism. The results of the present study indicated that CCAT2 regulated the expression of transcription factor 7-like 2 (TCF7L2) by targeting microRNA (miR)-217, and regulated the Wnt/ $\beta$ -catenin signaling pathway to control PCa. Therefore, the present study suggested that CCAT2 may display a pivotal role during PCa carcinogenesis, and may serve as an early diagnostic and molecular therapeutic target for PCa.

## Materials and methods

**Patient samples.** PCa tissues and adjacent normal prostate tissues (5-cm from the resection margin and pathologically confirmed) were obtained from 18 male patients (mean age, 60.8 years; age range, 50-72 years) who attended The Fifth Hospital of Wuhan (Wuhan, China) between March 2018 and November 2018. The patients had not received local or systemic treatment before surgery. Following prostatectomy, all specimens were collected within 20 min and immediately stored in liquid nitrogen until RNA extraction. The present study was approved by the Evaluation Committee of The Fifth Hospital of Wuhan (approval no. 2018-k-003). The present study carefully followed the ethical and procedural rules of clinical research, and obtained written informed consent from each patient before surgery.

**Cell culture.** The human PCa PC3 and DU145 cell lines, and human prostate epithelial RWPE-1 cell line were purchased from American Type Culture Collection. PC3 and DU145 cells were cultured in DMEM (HyClone; Cytiva) containing 10% fetal bovine serum (HyClone; Cytiva), 100  $\mu$ g/ml penicillin and 100  $\mu$ g/ml streptomycin. RWPE-1 cells were cultured in keratinocyte serum-free medium (cat. no. 10724; Gibco; Thermo Fisher Scientific, Inc.) containing 50  $\mu$ g/ml bovine pituitary extract L-glutamine and 5 ng/ml epidermal growth factor. All cells were cultured at 37°C with 5% CO<sub>2</sub>. Cells were periodically passaged to maintain exponential growth and passage two cells were used for subsequent experiments.

**Small interfering (si)RNA transfection.** An siRNA targeting CCAT2 (si-CCAT2) and the control siRNA [si-negative control (NC)] were constructed by Shanghai GenePharma Co., Ltd. The siRNA sequences used were as follows: si-CCAT2, 5'-GUGCAACUCUGCAAUUAU-3'; and si-NC, 5'-AATGGACAACCTGGTCGTGGAC-3'. A miR-217 mimic (miR-217) and its corresponding NC (miR-NC), and a miR-217 inhibitor and its corresponding NC (NC inhibitor) were purchased from Shanghai GenePharma Co., Ltd. Human *TCF7L2* cDNA (1,809 bp; Shanghai GeneChem Co., Ltd.) was inserted into a pcDNA3.1 plasmid (Shanghai GeneChem Co., Ltd.) to form *TCF7L2* overexpression plasmids. DU145 and PC3 cells were seeded (5 $\times$ 10<sup>5</sup> cells/well) into 6-well plates for 1 day prior to transfection. At 70% confluence, these molecular productions were transfected into DU145 and PC3 cells using Lipofectamine<sup>®</sup> 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The transfection concentrations were 10  $\mu$ mol for si-CCAT2 or siNC, and 50 nM for miR-217 mimic/inhibitor, their NCs or *TCF7L2* overexpression plasmids. Following incubation at 37°C for 48 h, cells were harvested and used for subsequent experiments.

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).** RT-qPCR was used to detect the mRNA expression levels of CCAT2 in PCa and adjacent non-cancerous tissues, as well as DU145 and PC3 cells. Total RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Isolated RNA (2  $\mu$ g) was reverse transcribed into cDNA using a reverse transcription kit (DRR037A, Takara Biotechnology Co., Ltd.) at 37°C for 15 min and 85°C for 5 sec. Subsequently, qPCR was performed using SYBR Green (Takara Biotechnology Co., Ltd.) and the Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The following primer pairs were used for qPCR: CCAT2 forward, 5'-ACTGGGAATGGAGGAAGA-3' and reverse, 5'-TGAGAAAGGATTGAGGGAAAAG-3'; TCF7L2 forward, 5'-ACGAGGGCGAACAGGAGGAG-3' and reverse, 5'-TGGGCGAGAGCGATCCGTTG-3'; miR-217 forward, 5'-GCGTACTGCATCAGGAAGTATTGGA-3' and reverse, 5'-GGGCACACAAAGGCAACTTTTGT-3'; and U6 forward, 5'-TGCGGGTGCTCGCTTCGCAGC-3' and reverse, 5'-CCA GTGCAGGGTCCGAGGT-3'. The following thermocycling conditions were used for qPCR: An initial step of 95°C for 60 sec, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing and extension at 59°C for 40 sec, and hold at 72°C for 15 sec. Expression levels were quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (38) and normalized to the internal reference gene U6. RT-qPCR was performed in at least triplicate.

**Cell proliferation assay.** Cell proliferation was assessed using the Cell Counting Kit-8 assay (CCK8; Dojindo Molecular Technologies, Inc.). DU145 and PC3 cells were seeded (5 $\times$ 10<sup>3</sup> cells/well) into 96-well plates after transfection and incubated at 37°C with 5% CO<sub>2</sub>. Cells were cultured at 37°C for 0, 24, 48, 72 and 96 h. Subsequently, ~20  $\mu$ l CCK8 reagent was added to each well and incubated for 2 h at 37°C in the dark. Then, 100  $\mu$ l dimethyl sulfoxide was added to dissolve the purple formazan. The absorbance of each well was

measured at a wavelength of 450 nm using a SpectraMax M3 microplate reader (Molecular Devices, LLC). The CCK-8 assay was performed in triplicate.

**Flow cytometry and cell cycle analysis.** DU145 and PC3 cells ( $3 \times 10^5$  cells/ml) were transfected/treated with si-CCAT2, si-NC or si-CCAT2 + 20 mmol/l lithium chloride (LiCl; Sigma-Aldrich Merck KGaA) at 37°C for 24 h. After 48 h, cells were centrifuged at  $1,000 \times g$  for 5 min at room temperature, washed with PBS and fixed with pre-cooled 70% ethanol at 4°C overnight. Subsequently, cells were stained at room temperature for 30 min with propidium iodide (PI; Sigma-Aldrich; Merck KGaA) staining buffer containing 500  $\mu\text{g/ml}$  PI and 100  $\mu\text{g/ml}$  RNase A at 37°C for 30 min in the dark. Cell cycle distribution was measured using a FACSCalibur flow cytometer (BD Biosciences), and cell cycle profiles were generated using ModFit software (v3.0; BD Biosciences). Cell cycle distribution is presented as the percentage of cells in the  $G_0/G_1$ , S and  $G_2/M$  phases. Flow cytometry was performed in triplicate.

**Cell apoptosis assay.** DU145 and PC3 cells ( $3 \times 10^5$  cells/ml) were transfected with si-NC and si-CCAT2. Following transfection for 48 h, cells were collected and washed with PBS. Apoptotic cells were detected using the Annexin V-FITC/PI Apoptosis Detection kit (BioTime, Inc.), according to the manufacturer's protocol. Cells were stained with Annexin V and PI at 37°C for 15 min in the dark. Apoptotic cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and BD FACSuite software (v1.0; BD Biosciences).

**Migration and invasion assay.** The migratory ability of DU145 and PC3 cells transfected with si-CCAT2 or si-NC was assessed using a 24-well Transwell chamber (BD Biosciences). For migration assays, PCa cells ( $1 \times 10^5$  cells) with serum-free DMEM were plated into the upper chambers, and DMEM containing 10% FBS (HyClone; Cytiva) was plated into the lower chambers. Cells were incubated at 37°C with 5%  $\text{CO}_2$  for 24 h. Following incubation, the upper surface of the Transwell membrane was wiped to move non-migratory cells. Migratory cells on the lower surface of the Transwell membranes were fixed in 4% paraformaldehyde at 4°C for 20 min, followed by staining with 0.1% crystal violet for 15 min at 25°C. Stained cells were observed in five random fields using a light microscope (magnification,  $\times 20$ ). To assess cell invasion, artificial basement membrane Matrigel® (BD Biosciences) was pre-coated at 37°C overnight on the bottom of the Transwell culture chamber.

**Western blot analysis.** Total protein was extracted from the transfected DU145 and PC3 cells using RIPA buffer (Aladdin Industrial Corporation) supplemented with 1% protease inhibitors (cat. no. P2850; Sigma-Aldrich; Merck KGaA) on ice for 30 min. Total protein was quantified using the bicinchoninic acid assay (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Equal amounts of protein (40  $\mu\text{g/lane}$ ) were separated via 10% SDS-PAGE and transferred onto a 0.22- $\mu\text{m}$  PVDF membrane (EMD Millipore). Following blocking at room temperature for 1 h

with 5% skim milk, the membrane was incubated overnight at 4°C with primary antibodies targeted against the following:  $\beta$ -catenin (1:1,000; cat. no. ab32572; Abcam), Cyclin D1 (1:1,000; cat. no. ab134175; Abcam), c-Myc (1:1,000; cat. no. ab39688; Abcam), GAPDH (1:1,000; cat. no. ab8245; Abcam), E-cadherin (1:200; cat. no. ab219332; Abcam), N-cadherin (1:200; cat. no. ab12221; Abcam), Vimentin (1:1,000; cat. no. ab137321; Abcam) and TCF7L2 (1:1,000; cat. no. ab76151; Abcam). Following incubation with the primary antibodies overnight, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse; 1:2,000; cat. no. abs20001; Absin Bioscience, Inc., or goat anti-rabbit; 1:5,000; cat. no. ab6721; Abcam) for 2 h at 37°C. Protein bands were visualized using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.). Protein expression levels were quantified using ImageJ software v1.48 (National Institutes of Health). The relative quantification was normalized to the control (such as si-NC).

**Bioinformatics prediction and dual-luciferase reporter assay.** The potential targets of CCAT2 and miR-217 were predicted using RNA Hybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/>) (39). The wild-type (WT) and mutant (MUT) miRNA binding site sequence of CCAT2 and TCF7L2 were constructed by Shanghai GeneChem Co., Ltd. DU145 and PC3 cells ( $1 \times 10^5$  cells) were co-transfected with CCAT2-WT, CCAT2-MUT, TCF7L2-WT or TCF7L2-MUT and miR-217 mimic or NC mimic using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 10 ng *Renilla* (Promega Corporation) was used as the internal control. After transfection for 48 h at 37°C, luciferase activities were assessed using a Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol, and the activity was normalized with *Renilla* luciferase activity. The dual-luciferase reporter assay was performed in triplicate.

**TOPflash assay.** PCa cells ( $1 \times 10^5$ ) were co-transfected with 250 ng WNT signaling luciferase reporter constructs (TOPflash) (Shanghai GeneChem Co., Ltd.) and 25 ng *Renilla* luciferase vector (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. At 48 h post-transfection at 37°C, luciferase activities were assessed using a Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activities were normalized to *Renilla* luciferase activities. The TOP values were calculated as the relative luciferase activity. The TOPflash assay was performed in triplicate.

**Statistical analysis.** Statistical analyses were performed using SPSS software (v21.0; IBM Corp.). Data are expressed as the mean  $\pm$  standard deviation of at least three independent experiments. Differences between the paired data were analyzed by a paired Student's t-test. Comparisons between different groups were performed using one-way ANOVA followed by Tukey's multiple comparisons test.  $P < 0.05$  was considered to indicate a statistically significant difference.

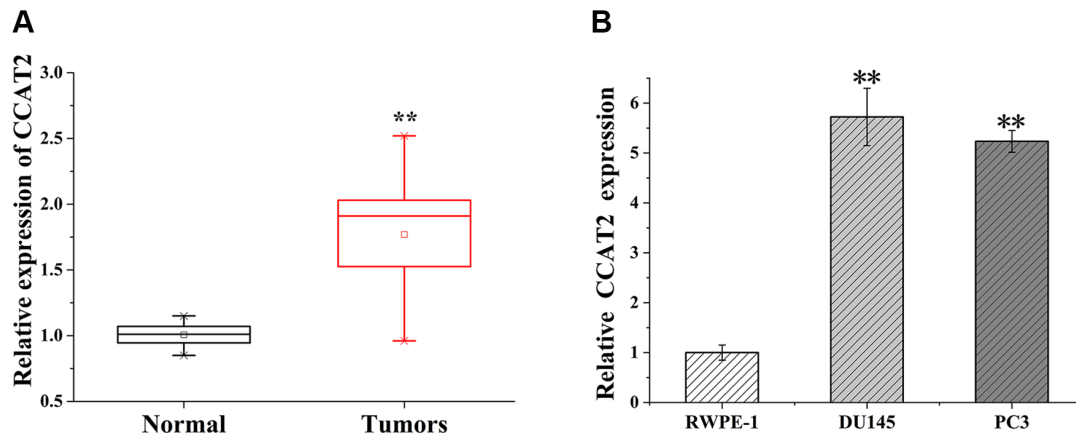


Figure 1. CCAT2 expression is upregulated in PCa tissues and cell lines. (A) Relative expression levels of CCAT2 in 18 paired PCa and adjacent non-tumor tissues were detected by reverse transcription-quantitative PCR. \*\* $P < 0.01$  vs. Normal. (B) CCAT2 expression levels in two prostate cancer cell lines (DU145 and PC3) and the human prostate epithelial cell line (RWPE-1). \*\* $P < 0.01$  vs. RWPE-1. Data are presented as the mean  $\pm$  SD ( $n=3$ ). CCAT2, colon cancer associated transcript 2; PCa, prostate cancer.

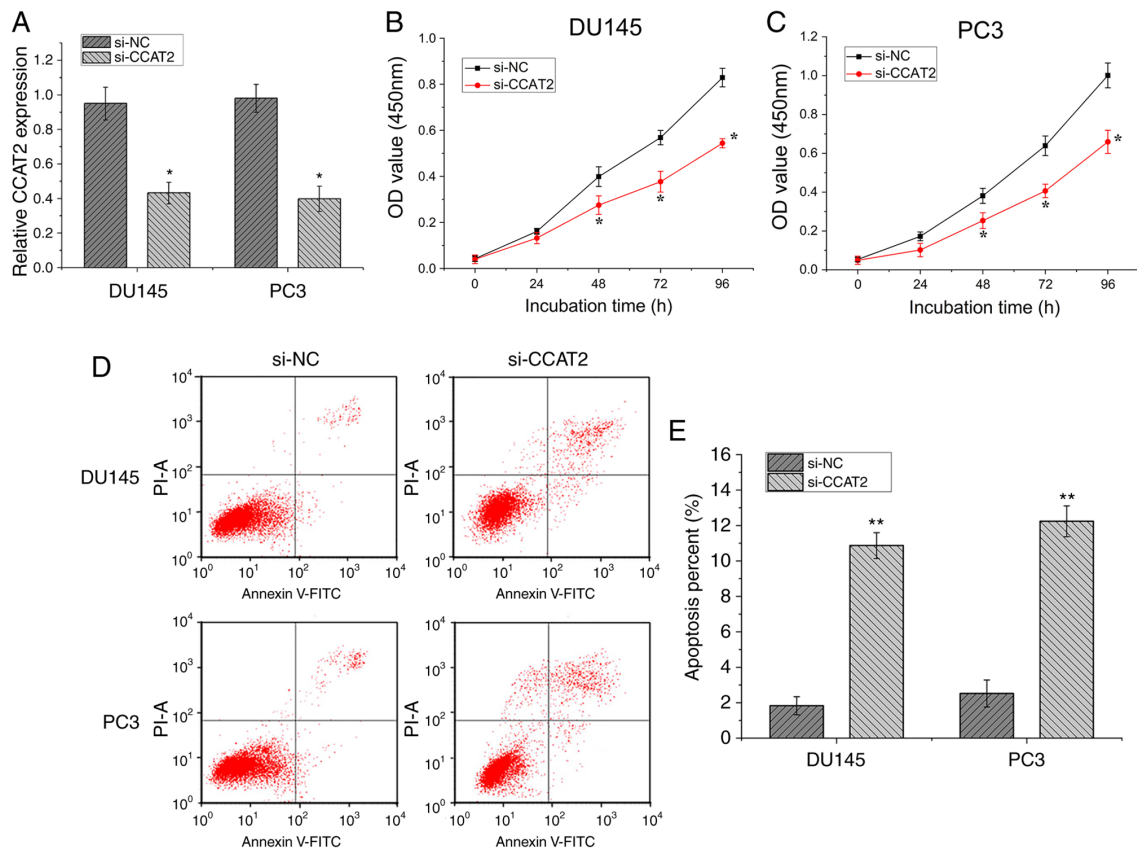


Figure 2. Effect of CCAT2 on PCa cell proliferation and cell apoptosis. (A) mRNA expression levels of CCAT2 in DU145 and PC3 cells to determine the transfection efficiency of siRNA. The Cell Counting Kit-8 assay assessed cell proliferation of (B) DU145 and (C) PC3 cells following si-CCAT2 and si-NC transfection. (D) DU145 and (E) PC3 cell apoptosis following si-NC and si-CCAT2 transfection was determined using an Annexin V/PI apoptosis detection kit. Data are presented as the mean  $\pm$  SD ( $n=3$ ). \* $P < 0.05$  and \*\* $P < 0.01$  vs. si-NC. CCAT2, colon cancer associated transcript 2; PCa, prostate cancer; si, small interfering RNA; NC, negative control; PI, propidium iodide; OD, optical density.

## Results

*CCAT2 is upregulated in PCa tissues and cell lines.* RT-qPCR was performed to detect the expression of lncRNA CCAT2 in paired tissue samples obtained from 18 patients with PCa. CCAT2 expression levels were significantly higher in PCa tissues compared with adja-

cent non-tumor tissues ( $P < 0.01$ ; Fig. 1A). Subsequently, RT-qPCR was performed to detect the expression levels of CCAT2 in the human prostate epithelial RWPE-1 cell line and the human PCa DU145 and PC3 cell lines. The results revealed that CCAT2 expression levels were significantly higher in DU145 and PC3 cells compared with RWPE-1 cells ( $P < 0.01$ ; Fig.1B). The results indicated that abnormal

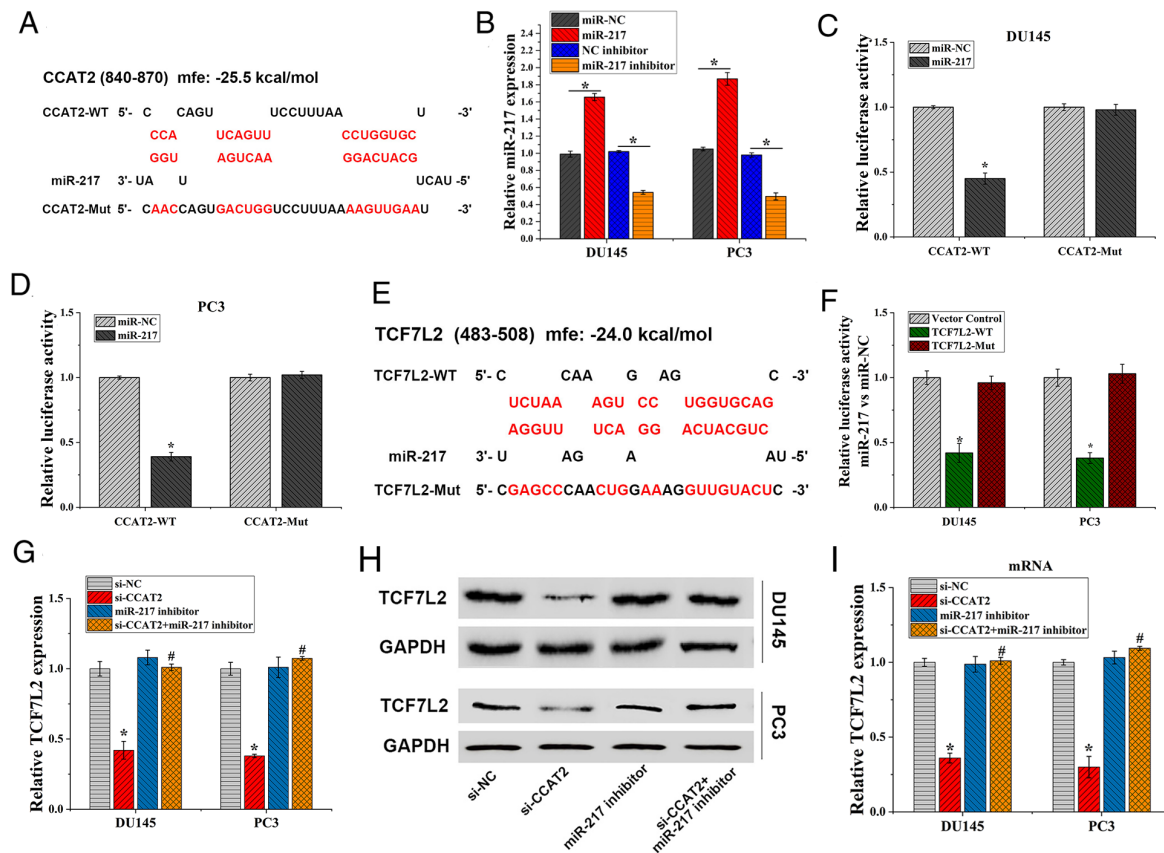


Figure 3. CCAT2 regulates TCF7L2 expression by binding to miR-217. (A) Schematic illustration of the putative binding sites between CCAT2 and miR-217, which were predicted by RNA Hybrid. (B) mRNA expression levels of miR-217 in DU145 and PC3 cells to determine the transfection efficiency of miR-217 mimics and miR-217 inhibitor. \* $P < 0.05$ . Dual-luciferase reporter assays were performed to verify the interaction between miR-217 and CCAT2 in (C) DU145 and (D) PC3 cells. \* $P < 0.05$  vs. miR-NC. (E) The binding site between miR-217 and TCF7L2 was predicted by RNA Hybrid. (F) Dual-luciferase reporter assays were performed to verify the interaction between miR-217 and TCF7L2. \* $P < 0.05$  vs. vector control. (G) Reverse transcription-quantitative PCR was performed to detect the relative mRNA expression levels of TCF7L2 in the different groups. \* $P < 0.05$  vs. si-NC; # $P < 0.05$  vs. si-CCAT2. (H and I) Western blotting was performed to measure relative TCF7L2 expression. \* $P < 0.05$  vs. si-NC; # $P < 0.05$  vs. si-CCAT2. Data are presented as the mean  $\pm$  SD ( $n = 3$ ). CCAT2, colon cancer associated transcript 2; miR, microRNA; TCF7L2, transcription factor 7 like 2; si, small interfering RNA; NC, negative control; Mut, mutant; WT, wild-type; mfe, minimum free energy.

CCAT2 expression may be associated with the pathogenesis of PCa and may serve a crucial role during the progression of the disease.

**CCAT2 regulates cell proliferation and cell apoptosis in PCa.** To further investigate the role of CCAT2 during the progression of PCa, CCAT2 expression was knocked down in DU145 and PC3 cells using a specific siRNA to determine its effect on the biological function of PCa cells. The expression level of CCAT2 mRNA in transfected si-CCAT2 cells was significantly lower compared with cells transfected with si-NC control siRNA (Fig. 2A;  $P < 0.05$ ). The CCK-8 assay indicated that DU145 (Fig. 2B) and PC3 (Fig. 2C) cells exhibited significantly reduced cell proliferation at 48, 72 and 96 h in the si-CCAT2 group compared with the si-NC group ( $P < 0.05$ ). Subsequently, flow cytometry was performed to examine the effect of CCAT2 on the PCa cell apoptosis (Fig. 2D). si-CCAT2 significantly promoted DU145 and PC3 cell apoptosis compared with si-NC ( $P < 0.01$ ; Fig. 2E). Collectively, the aforementioned results indicated that CCAT2-knockdown inhibited PCa cell proliferation and promoted PCa cell apoptosis.

**CCAT2 regulates TCF7L2 expression by binding to miR-217.** To further investigate the mechanism of action underlying CCAT2 during the development of PCa, RNA Hybrid was used to predict potential target miRs of CCAT2. The results indicated that CCAT2 bound to miR-217 with -25.5 kcal/mol binding energy (Fig. 3A). As shown in Fig. 3B, after PCa cells were transfected with miR-217 mimic and miR-217 inhibitor, the mRNA expression of miR-217 was significantly different from that of the NC, indicating successful transfection. To investigate the binding function of the genes, a dual-luciferase gene reporter assay was performed. In DU145 and PC3 cells, luciferase activities of CCAT2-WT were significantly reduced by miR-217 compared with miR-NC ( $P < 0.05$ ), but not that of the CCAT2-MUT (Fig. 3C and D). Moreover, the RNA Hybrid prediction analysis identified binding sites between miR-217 and TCF7L2, which displayed -24.0 kcal/mol binding energy (Fig. 3E). Furthermore, the dual-luciferase gene reporter assay indicated that miR-217 mimic inhibited luciferase activities in the TCF7L2-WT group, but exhibited no effect in the TCF7L2-MUT group (Fig. 3F). The results confirmed that TCF7L2 was a target gene of miR-217. In addition, CCAT2-knockdown significantly reduced the mRNA

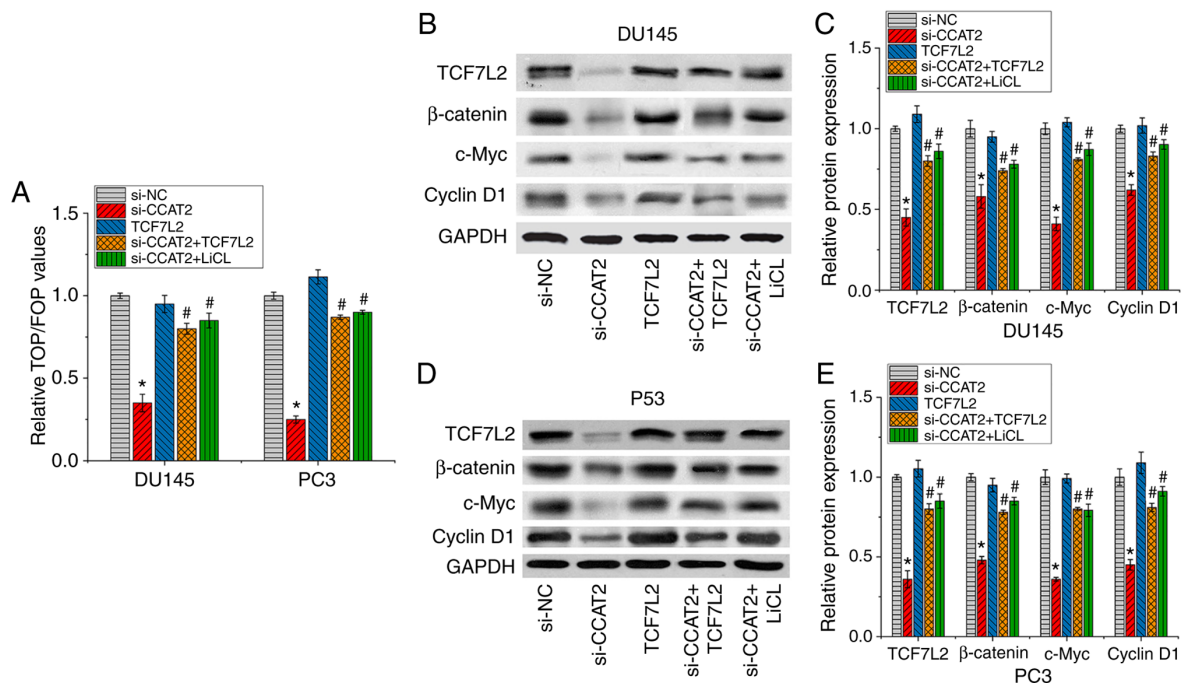


Figure 4. CCAT2-knockdown inhibits TCF7L2 via miR-217, thereby inhibiting the Wnt/β-catenin signaling pathway. PCa DU145 and PC3 cells were treated with si-NC, si-CCAT2, TCF7L2, si-CCAT2+TCF7L2 or si-CCAT2 + LiCl. (A) The TOPflash assay was used to measure Wnt/β-catenin signaling activity. Western blotting was performed to detect the expression levels of TCF7L2, β-catenin, c-Myc and CyclinD1 in PCa (B and C) DU145 and (D and E) PC3 cells. Data are presented as the mean ± SD (n=3). \*P<0.05 vs. si-NC; #P<0.05 vs. si-CCAT2. CCAT2, colon cancer associated transcript 2; TCF7L2, transcription factor 7 like 2; miR, microRNA; si, small interfering RNA; NC, negative control; LiCl, lithium chloride; PCa, prostate cancer.

(Fig. 3G) and protein (Fig. 3H and I) expression levels of TCF7L2. However, miR-217 inhibitor reversed the inhibitory effect of CCAT2-knockdown on TCF7L2 expression. The results suggested that CCAT2 regulated TCF7L2 expression by binding to miR-217.

*CCAT2-knockdown inhibits the Wnt/β-catenin signaling pathway by downregulating TCF7L2.* Wnt/β-catenin signaling can regulate a variety of developmental events, including proliferation and migration (40). In addition, it has been reported that lncRNA CCAT2 affects glioma, breast cancer and other tumors by regulating the Wnt/β-catenin pathway (35-37). TCF7L2 is an effector of the Wnt/E-catenin signaling pathway (41). Previous studies have indicated that TCF7L2 is involved in regulating the Wnt/β-catenin signaling pathway in a number of different types of cancer (42,43). Therefore, whether CCAT2 regulated PCa by increasing miR-217 expression and consequently inhibiting the TCF7L2-mediated Wnt/β-catenin signaling pathway was investigated. The TOPflash assay was performed to evaluate the activity of the Wnt/β-catenin signaling pathway in PCa cells. CCAT2-knockdown inhibited the Wnt/β-catenin signaling pathway and TCF7L2 overexpression increased the activity of the Wnt/β-catenin signaling pathway (Fig. 4A). In addition, TCF7L2 overexpression partially reversed the inhibitory effect of CCAT2 on the Wnt/β-catenin signaling pathway. Western blotting was performed to measure the protein expression levels of TCF7L2 and Wnt/β-catenin signaling pathway-related proteins β-catenin, c-Myc and Cyclin D1 following si-CCAT2 and TCF7L2 transfection in PCa cells. The western blotting results were consistent with the TOPflash assay results (Fig. 4B-E). Collectively, the results suggested

that CCAT2 regulated the progression of PCa by targeting miR-217 to inhibit TCF7L2 expression and the Wnt/β-catenin signaling pathway; therefore, this signaling pathway may serve as an intrinsic molecular mechanism by which CCAT2 regulates PCa.

*CCAT2-knockdown arrests PCa cell cycle and impairs cell migration and invasion in vitro.* Compared with si-NC cells, the percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase was significantly increased in si-CCAT2-transfected DU145 and PC3 cells (Fig. 5C and C). By contrast, the percentage of cells in the S phase was significantly decreased in si-CCAT2-transfected DU145 and PC3 cells compared with si-NC cells (Fig. 5C and D). Therefore, the results suggested that CCAT2-knockdown shifted the cell cycle from S phase to G<sub>0</sub>/G<sub>1</sub> phase. Transwell assays were performed to assess the role of CCAT2 in DU145 and PC3 cell migration and invasion. Compared with si-NC cells, the number of migratory cells was significantly reduced in the si-CCAT2 group, which indicated that CCAT2-knockdown reduced the migratory ability of PCa cells (P<0.05; Fig. 5G and H). CCAT2-knockdown also significantly inhibited the invasion of PCa cells compared with the si-NC group (P<0.05; Fig. 5I and J). Furthermore, epithelial-mesenchymal transition (EMT) is an important factor during cell migration and invasion. The western blotting results indicated that the expression levels of N-cadherin and vimentin were decreased, while E-cadherin expression levels were increased following CCAT2-knockdown in DU145 cells and PC3 cells (Fig. 5E and F). In summary, the results suggested that CCAT2-knockdown arrested PCa cell cycle, and inhibited PCa cell migration and invasion.

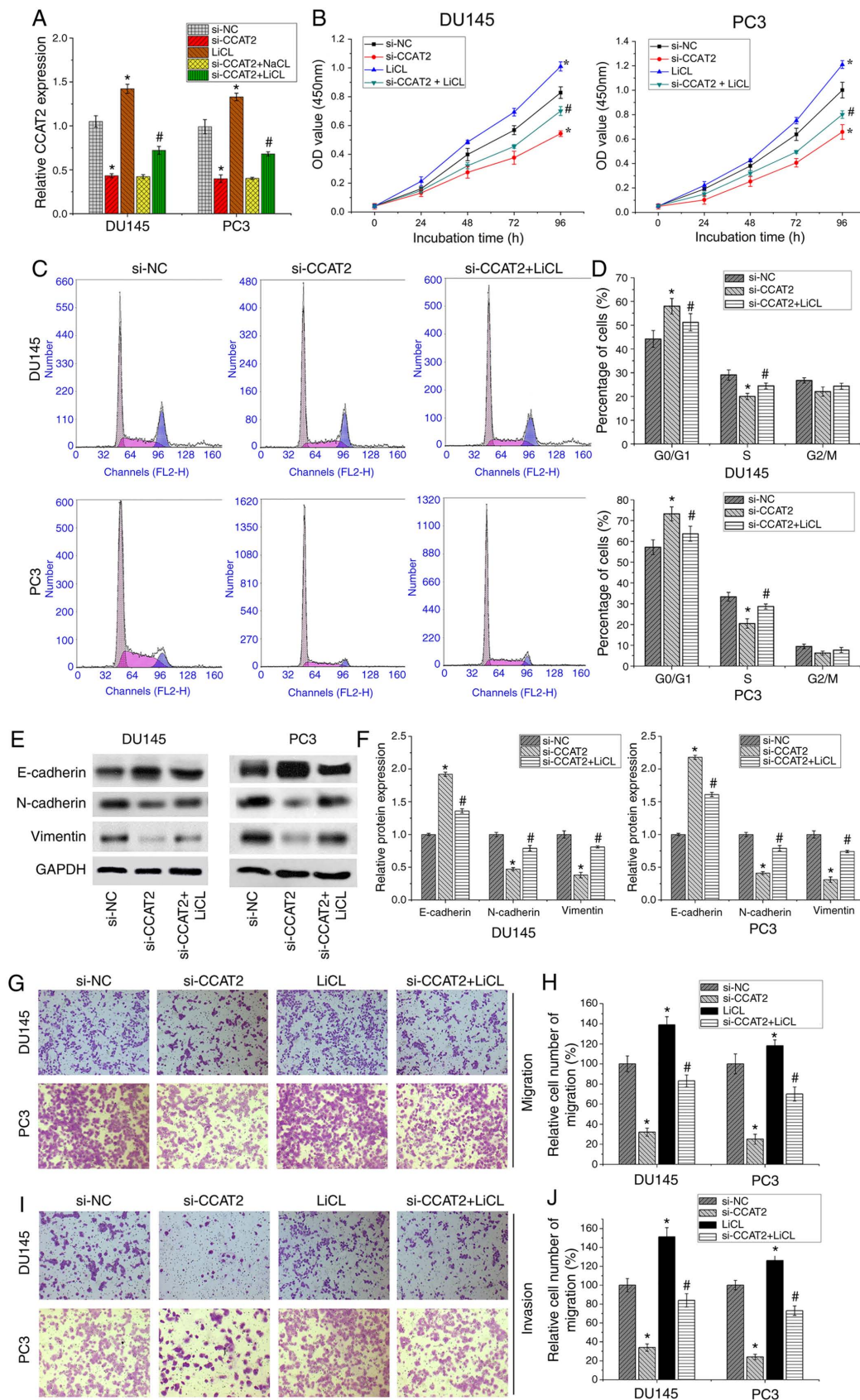


Figure 5. CCAT2 and Wnt signaling agonist LiCl affect PCa cell proliferation, cell cycle, migration and invasion. (A) CCAT2 expression was detected by reverse transcription-quantitative PCR. LiCl treatment enhanced CCAT2 expression. (B) The Cell Counting Kit-8 assay detected PCa cell proliferation following transfection with si-NC, si-CCAT2, LiCl or si-CCAT2 + LiCl. (C and D) Flow cytometry analysis of the cell cycle distribution of PCa cells following transfection with si-NC, si-CCAT2 or si-CCAT2 + LiCl. (E and F) Western blotting was performed to detect the expression of epithelial-mesenchymal transition-related proteins, including E-cadherin, N-cadherin and Vimentin. (G and H) Transwell migration assay in PCa cells transfected with si-NC, si-CCAT2, LiCl and si-CCAT2 + LiCl. Magnification, x200. (I and J) Transwell invasion assay in PCa cells transfected with si-NC, si-CCAT2, LiCl or si-CCAT2 + LiCl. Magnification, x200. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs. si-NC; #P<0.05 vs. si-CCAT2. CCAT2, colon cancer associated transcript 2; LiCl, lithium chloride; PCa, prostate cancer; si, small interfering RNA; NC, negative control; OD, optical density.

*Wnt/β-catenin signaling activation partly restores CCAT2-mediated effects on PCa cells.* To further verify whether CCAT2 controlled PCa cell proliferation, migration and invasion by regulating Wnt/β-catenin signaling, 20 mmol/l LiCl was used to activate Wnt/β-catenin signaling. PCa DU145 and PC3 cell lines were divided into the following groups: i) si-NC control group; ii) si-CCAT2 group; iii) Wnt/β-catenin signal activator LiCl group; and iv) si-CCAT2 combined with LiCl. LiCl not only activated the Wnt/β-catenin signaling pathway, increased the recruitment of β-catenin, and promoted the expression of the classical downstream genes of the Wnt/β-catenin signaling pathway, Cyclin D1 and c-Myc (Fig. 4), but also promoted cell proliferation (Fig. 5B), migration (Fig. 5G and H) and invasion (Fig. 5I and J).

The western blotting results indicated that the combined treatment of si-CCAT2 and LiCl partially increased the transcriptional activity of the Wnt/β-catenin signaling pathway, increased the recruitment of β-catenin, and increased the expression of downstream genes CyclinD1 and c-Myc compared with si-CCAT2. However, si-CCAT2 and LiCl did not restore the activation level of the Wnt/β-catenin signaling pathway to level of the LiCl group (Fig. 4).

In addition, the RT-qPCR results indicated that LiCl treatment significantly enhanced CCAT2 expression (Fig. 5A). An *in vitro* cell assay further suggested that DU145 and PC3 cells treated with si-CCAT2 and LiCl displayed significantly increased cell proliferation, migration and invasion compared with si-CCAT2 (Fig. 5B and G-J). Regarding the cell cycle, LiCl partially reversed si-CCAT2-mediated S phase arrest in PCa cells. Furthermore, following treatment with LiCl, CCAT2-knockdown-mediated effects on EMT-related protein expression were partially reversed (Fig. 5E and F). The results indicated that activation of the Wnt/β-catenin signaling pathway partially restored CCAT2-mediated malignant biological behavior in PCa cells. Furthermore, the results suggested that CCAT2 controlled the progression of PCa via the Wnt/β-catenin signaling pathway.

## Discussion

Increasing evidence has suggested that lncRNAs serve an important role in various malignancies (44,45). Previous studies have suggested that lncRNAs may serve as novel tumor biomarkers, providing a new approach to the early diagnosis and treatment of cancer (46). As a novel lncRNA, CCAT2 was first reported to be highly overexpressed in colorectal cancer, which affects Wnt signaling activity, and promotes tumor metastasis and chromosomal instability (47). The carcinogenic effect of CCAT2 in human cancer has been confirmed in multiple previous studies (48-50). It has been reported that CCAT2 is upregulated in ovarian, lung and cervical cancer, as well as in esophageal squamous cell carcinoma, and high expression of CCAT2 is often associated with tumor progression and poor clinical outcomes (20,49,50). It has been reported that CCAT2 is also upregulated in PCa (24); however, to the best of our knowledge, the molecular regulation mechanism underlying lncRNA CCAT2 during PCa has not been previously reported.

In the present study, RT-qPCR detection was performed on 18 paired PCa and paracancerous tissues, as well as on PCa cell lines (DU145 and PC3). Consistent with previous

studies, the expression level of CCAT2 in PCa tissues and cell lines was significantly higher compared with proximal prostate tissue and normal human prostate epithelial RWPE-1 cells. To further investigate the clinical role of CCAT2 during PCa, the functions of CCAT2 in PCa were investigated using CCK-8, cell cycle, cell apoptosis and Transwell assays. The present experiments demonstrated that inhibition of CCAT2 expression arrests the cell cycle and induces cell apoptosis to inhibit PCa cell proliferation, migration and invasion, which indicated that CCAT2 displayed an oncogene role during PCa.

Previous studies have reported that lncRNAs may affect the biological behavior of malignant tumors via various signaling pathways. For example, lncRNA HOXA distal transcript antisense RNA regulates osteosarcoma by activating the Wnt/β-catenin signaling pathway (51). Furthermore, lncRNA small nucleolar RNA host gene 20 promotes glioblastoma and tumor progression by activating the PI3K/Akt/mTOR signaling pathway (52) and lncRNA colorectal neoplasia differentially expressed promotes liver cancer by enhancing the Wnt/β-catenin signaling pathway (53). Several studies have demonstrated that the typical Wnt/β-catenin signaling pathways are involved in a variety of cellular processes, including cell proliferation, autophagy, cell cycle, cell differentiation and tumorigenesis (54-57). When Wnt/β-catenin is activated, cytoplasmic β-catenin protein is upregulated, leading to accumulation and nuclear transfer, which can result in activation of the transcription of downstream target genes, including CyclinD1, c-Myc and matrix metalloproteinases (58,59).

CCAT2 is a downstream target of the Wnt signaling pathway (46). A number of studies have reported that CCAT2 affects breast and non-small cell lung cancer, as well as glioma, renal cell carcinoma and other tumors by activating the Wnt/β-catenin signaling pathway (35-37). To further study the regulatory mechanism underlying CCAT2 during PCa, luciferase reporter assays were performed to verify the interaction between CCAT2 and miR-217, as well as the interaction between miR-217 and TCF7L2. TCF7L2 is a key member of the Wnt/β-catenin signaling pathway, representing a central factor in cell proliferation, invasion and death (41,60). The results of the TOPflash assay and western blotting indicated that CCAT2-knockdown inhibited the expression of TCF7L2, which led to a decrease in β-catenin activity in the Wnt/β-catenin signaling pathways in both PCa cell lines. In addition, the downstream targeting genes Cyclin D1 and c-Myc were also downregulated following CCAT2-knockdown. Therefore, the results indicated that si-CCAT2 may regulate PCa by inhibiting the Wnt/β-catenin signaling pathway via TCF7L2.

To further identify the potential molecular mechanisms underlying CCAT2 during PCa, cells were treated with si-CCAT2 and LiCl, a Wnt/β-catenin signaling pathway activator (34,61). LiCl treatment not only reversed CCAT2-knockdown-mediated effects on the expression of β-catenin, CyclinD1 and c-Myc in the Wnt/β-catenin signaling pathway, but also reversed the inhibitory effects of CCAT2-knockdown on PCa cell proliferation, cell cycle, migration and invasion. It was further suggested that CCAT2 inhibited the expression of N-cadherin and vimentin, and promoted the expression of E-cadherin by inhibiting the Wnt/β-catenin signaling pathway, thereby inhibiting cell EMT. Therefore, the results indicated that CCAT2 regulated PCa via the Wnt/β-catenin signaling pathway.

In conclusion, the present study extended the existing knowledge of the molecular mechanisms underlying CCAT2-mediated regulation of PCa. The results indicated that CCAT2 regulated the expression of TCF7L2 by targeting miR-217, which consequently regulated Wnt/ $\beta$ -catenin signaling to alter the biological behavior of PCa cells. Therefore, CCAT2 may serve as a novel molecular therapeutic target for PCa; however, further studies with larger sample sizes are required to verify the results of the present study.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

PH performed the experiments and wrote the manuscript. GX and GJ collected the samples and acquired the data. GX and WG analyzed the data. YL assisted in the experiments and data processing. HL conceived the study and revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Evaluation Committee of The Fifth Hospital of Wuhan (approval no. 2018-k-003; Wuhan, China). Written informed consent was obtained from each patient.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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