LncRNA CCAT1 Promotes Prostate Cancer Cell Proliferation by Interacting with DDX5 and MIR-28-5P

Zonghao You1,2, Chunhui Liu1, Can Wang1, Zhixin Ling3, Yiduo Wang1, Yali Wang1, Minghao Zhang2, Shuqiu Chen1, Bin Xu1, Han Guan4, and Ming Chen1

Abstract

Accumulated evidence indicates that CCAT1 functions as an oncogene in the progression of a variety of tumors. However, little is known as to how CCAT1 impacts tumorigenesis in human prostate cancer. In this study, we found from The Cancer Genome Atlas and Memorial Sloan Kettering Cancer Center database that CCAT1 is highly upregulated in castration-resistant prostate cancer (CRPC) compared with androgen-dependent prostate cancer (ADPC). Higher level of CCAT1 leads to increased mortality in patients with CRPC. In vitro and in vivo studies show that CCAT1 promotes prostate cancer cell proliferation as well as the tumor growth of prostate cancer xenografts. Mechanistically, in cytoplasm, CCAT1 sponges MIR-28-5P to prevent the anticancer effect. In nucleus, CCAT1 acts as a scaffold for DDX5 (P68) and AR transcriptional complex to facilitate the expression of AR-regulated genes, thus stimulating CRPC progression. Our findings suggest that CCAT1 is an oncogenic factor in the progression of CRPC with different regulatory mechanisms in the nucleus and cytoplasm of cells.

Introduction

Prostate cancer is the most commonly diagnosed cancer among American men and second leading cause of cancer-related death in elderly men (1). Morbidity and mortality of prostate cancer have also shown a great increase over the past 10 years in China (2). Although androgen deprivation therapy (ADT) has become the first-line treatment for advanced prostate cancer since 1941, the biggest obstacle to prostate cancer treatment is that prostate cancer progresses to castration-resistant prostate cancer (CRPC) within 2 years after ADT, which accounts for the most cases of death for patients with advanced prostate cancer. Therefore, it is urgent to uncover the pathogenesis of prostate cancer progression and to develop corresponding therapeutic strategies.

Long noncoding RNAs (lncRNA), a class of nonprotein-coding transcripts longer than 200 nucleotides, were originally considered to be “noisy” transcripts. However, an increasing number of studies have confirmed the crucial functions of these “noisy transcripts” in chromatin remodeling, transcriptional, and posttranscriptional regulations (3–5). Significantly, aberrant expression of lncRNA plays an important role in a wide range of diseases including cancers, leading to abnormal cell proliferation, migration, invasion, and apoptosis (6, 7). For instance, lncRNA HOTAIR promotes metastasis in breast cancer by binding to the polycomb repressive complex (PRC2) and represses transcription of HOX loci (3). lncRNA LUCAT1 facilitates tumor progression by regulating the ubiquitination and stability of DNA methyltransferase-1 (DNMT1) in esophageal squamous cell carcinoma (8). Through high-throughput RNA sequencing, researchers have identified lncRNAs with oncogenic or tumor-suppressive roles that are involved in the pathogenesis of prostate cancer (5, 9–11).

LncRNA colon cancer associated transcript-1 (CCAT1), located on chromosome 8q24.21, was first identified as an oncogene in colorectal cancer by Nissan and colleagues (12). The overexpression of CCAT1 was recently proven to activate the initiation and progression in a variety of cancers (13–16) by sponging miRNAs such as MIR-490-3P, MIR-218-5P, and MIR-7. However, the biologic functions of CCAT1 and molecular mechanisms underlying CCAT1’s role in prostate cancer remain unclear. With RNA pulldown and consequent miRNA arrays and protein sequencing, we identified a direct binding of CCAT1 to the Asp-Glu-Ala-Asp (DEAD) box helicase 5 (DDX5) in ADPC cells (LNCaP) and the interaction between CCAT1 and MIR-28-5P in CRPC cells (Du145).

DDX5 (P68), one of the large DEAD-box family, is an ATP-dependent RNA helicase that has been identified as a coactivator for transcriptional factors such as the androgen receptor (AR; ref. 17), estrogen receptor (18), P53 (19), and C-FOS (20). Overexpression of DDX5 leads to the development of colorectal
tumors (21), lung cancer (22), breast cancer (23), leukemia (24), and prostate cancer (25). According to Clark and colleagues (17, 25), DDX5 is recruited to the AR transcriptional complex and required for the transcriptional regulation of AR-targeted genes. In our study, we found that CCAT1 is significantly upregulated in CRPC tissues, compared with ADPC tissues. In addition, CCAT1 regulates cellular proliferation both in vitro and in vivo. Mechanistic analyses found that CCAT1 exhibits different regulatory mechanisms in cellular nuclei and cytoplasm.

Materials and Methods
Reanalyses of The Cancer Genome Atlas and Memorial Sloan Kettering Cancer Center prostate cancer databases
We retrieved and reanalyzed the original IncRNA expression and clinical data from The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov) and the Memorial Sloan Kettering Cancer Center (MSKCC; www.mskcc.org) databases to investigate clinical relevance of CCAT1 with respect to the pathologic traits of patients.

Patient samples
ADPC tissues (n = 8) were obtained from early-stage patients who underwent radical prostatectomy but never received any other treatment. Three had a Gleason score <7, 3 with a Gleason score = 7, and 2 with a Gleason score >7. CRPC specimens (n = 4) were obtained from patients who were diagnosed as CRPC for their serum prostate-specific antigen (PSA) levels continued to increase after 2 years of ADT. All of the patients had a Gleason score >8. For each specimen, a portion of tumor tissue was confirmed by PSA staining and the samples with >60% tumor involvement were included in the study. The study was approved by the Ethics Committee of the Affiliated Zhongda Hospital of Southeast University (Nanjing, China). All patients received the written informed consent and with their consent, tumor samples were obtained. Meanwhile, the investigators obtained the informed written consent from the subjects.

Cell culture
We obtained prostate cancer cell lines PC3, Du145, and LNCaP from ATCC. Short tandem repeat (STR) confirmation was conducted by GeneChem. Cells were cultured in RPMI1640 medium ( Gibco; Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO2. We used cells from passages 8 to 15 in our experiments.

RNA extraction and qRT-PCR
Total RNA was extracted from cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific). For qRT-PCR, RNA was reverse transcribed to cDNA using Hiscrypt II First-Strand cDNA Synthesis Kit (Vazyme Biotech Co.). Real-time PCR analyses were performed with SYBR Green (Vazyme Biotech Co.). Results were normalized to the expression of GAPDH or U6. Primers were chemically synthesized by Sangon Biotech and their sequences are listed in Supplementary Table S1.

Subcellular fractionation location
The separation of the nuclear and cytosolic fractions was performed using a PARIS Kit (AM1921; Life Technologies) according to the manufacturer's instructions.

Oligonucleotides, plasmids, lentivirus
On the basis of the miRBase database, MIR-28-5P mimics, MIR-125a-3P mimics, MIR-125b-5P mimics, negative control for miRNA (miR-NC), anti-MIR-28-5P oligos (MIR-28-5P inhibitor), anti-MIR-125a-3P oligos (MIR-125a-3P inhibitor), anti-MIR-125b-5P oligos (MIR-125b-5P inhibitor), and negative control anti-miRNA (anti-miR-NC) were designed and synthesized by GeneChem. Full-length CCAT1 was synthesized and cloned into plasmid GV219 (GeneChem) to generate the CCAT1 overexpression vector. Empty plasmid GV219 (GeneChem) was used as the control. The reverse complementary sequence for mature CCAT1 was synthesized and subcloned into plasmid GV248 vector (GeneChem) to generate the inhibitory CCAT1 expression construct named Lv-anti-CCAT1. Empty plasmid GV248 was used as a control (Lv-NC). Sequences were listed in Supplementary Table S1.

Transfection of cell lines
We transfected oligonucleotides and plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Lentivirus was used to infect cells in the presence of polybrene. PC3 and Du145 cells were transfected with Lv-anti-CCAT1 or Lv-NC, and LNCaP cells were transfected for stable overexpression of CCAT1 vector or with control vector. Du145 cells were transiently transfected using miRNA mimics or miRNA inhibitors. The efficiency of knockdown and overexpression was determined by qRT-PCR.

Cell proliferation analysis
Cell viability was tested with a CCK-8 Kit (KeyGene Biotech) according to the manufacturer's instructions. For colony formation assay, 1,000 transfected cells were seeded in 6-well plates and maintained for 12 days at 37°C in 5% CO2 in appropriate medium containing 10% FBS, during which the medium was replaced every 4 days. Cells were washed twice with PBS. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 minutes. Colonies containing more than 50 cells were counted.

Flow cytometric analysis of cell cycle and apoptosis
For cell-cycle assay, we collected transfected cells and fixed them with 75% cold ethanol at –20°C overnight. Cells were stained with 50 μg/mL of propidium iodide (PI) in a 1 mg/mL RNase solution for 30 minutes at 4°C. Cell-cycle distributions were analyzed using a FACS flow cytometer (BD Biosciences). Apoptosis was conducted with an Annexin V-FITC/PI Apoptosis Detection Kit or an Annexin V-APC/PI Apoptosis Detection Kit (KeyGene Biotech) according to the manufacturer's instructions. Cell Quest Pro Software (BD Biosciences) was used to analyze cellular apoptosis.

Western immunoblotting analysis
RIPA lysis buffer (KeyGene Biotech) was used to lyse cells and extract proteins. After boiled, 40-μg protein samples were separated by 10% SDS-PAGE and were transferred onto polyvinylidene fluoride membrane. 5% nonfat milk was used to block nonspecific binding, followed by primary antibody incubation at 4°C overnight. After horseradish peroxidase (HRP)-conjugated secondary antibodies incubation at room temperature for 1 hour, membrane was developed by ECL. Western Blotting
Substrate (Thermo Fisher Scientific). The primary antibodies included anti-P68 (1:1,000; Abcam), anti-P53 (1:1,000; Abcam), anti-CASPASE-3 (1:1,000; Abcam), anti-P21 antibody (1:1,000; Abcam), and anti-P53 antibody (1:1,000; Abcam). Anti-β-ACTIN (1:2,000; Abcam) and anti-GAPDH (1:5,000; Beyotey) were used as the loading controls.

**Tumorigenicity assay in vivo**

All animal experiments were conducted with approval by Ethics Committee of Zhongda Hospital Affiliated to Southeast University, in accordance with the National Guidelines for the Health Use of Laboratory Animals. Six-week-old BALB/C nude mice were obtained from Shanghai SLAC Laboratories. PC3 (5 × 10⁶) cells, transfected with Lv-anti-CCAT1 or Lv-NC, were implanted subcutaneously in the back of mice. After 7 days of implantation, volume changes of tumors (calculated with the formula: length × width²/2) were recorded every 4 days. Mice were sacrificed and tumors were weighed and fixed for IHC staining by the end of the experiment.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were performed using an EZ-CHIP KIT according to the manufacturer’s instructions (Millipore). Briefly, cells were crosslinked with 37% (v/v) formaldehyde for 10 minutes at room temperature and the reaction was terminated with 125 mmol/L glycine treatment for 10 minutes. The extracted chromatin was digested, sonicated, and fragmented into 150 to 300 bp. Chromatin extracts were immunoprecipitated with anti-P68 and anti-IgG antibodies on Protein-A/G-Septasose beads. After washing, elution, and de-crosslinking, qRT-PCR was performed to quantify the immunoprecipitated DNA. The ChIP primer sequences are listed in Supplementary Table S1.

**RNA immunoprecipitation assays**

We performed RNA immunoprecipitation (RIP) experiments using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions. Briefly, cells were lysed in RIP lysis buffer, cleared lysates were incubated with RIP buffer containing protein A/G magnetic beads conjugated to human P68 antibody or the same type of IgG antibody at 4°C overnight. The coprecipitated RNA was then separated from the beads–antibody–RNA complexes for reverse transcription followed by PCR analysis.

**In vitro transcription assays and RNA pulldown assays**

CCAT1 plasmid DNA was linearized with restriction enzyme BamHI and XhoI. Biotin-labeled RNAs were in vitro transcribed with the Biotin RNA Labeling Mix and T7 SP6 RNA polymerase (AM1312; Invitrogen). Before purification with the RNeasy Mini Kit (Qiagen), biotin-labeled RNAs needed to be treated with RNase-free DNase I (Roche) to digest DNA templates in vitro transcription systems. LNCaP cell nuclear extraction (1 mg) was incubation with biotinylated RNA (100 pmol) at room temperature for 1 hour. Then, BSA-washed streptavidin agarose beads (100 μL) were added to each RNA–protein mixture and further rotary incubated at 4°C overnight. Beads were washed briefly three times and boiled in SDS sample buffer. The retrieved protein was separated using electrophoresis then silver-stained. The retrieved P68 protein was further validated by standard Western blot analysis.

**Dual luciferase reporter assay**

We cotransfected 2 × 10⁴ Du145 cells with pmirPGL-CCAT1-WT or -mut reporter plasmids (GeneChem), and with MIR-28-5P mimic or miR-NC. The relative luciferase activity was measured after 48 hours of transfection using the Dual-Luciferase Reporter Assay System (Promega), and normalized against Renilla luciferase activity.

**In situ hybridization and IHC staining**

The double (5′−3′) digoxigenin (DIG)-labeled CCAT1 probe (sequences: 5′-TACGTCATCCTTAGATCATCAGATGTGTCGAA−3′, 5′-GTAACGGCTGCGCTTCTGCACTAGAGATCCTTCGTCCTC−3′ and 5′-TCCAATCTGATCTCAATATGCTGTAGTCTGATAATTATT-3′) and U6 probe were obtained from Boster. Paraffin-embedded slides were dewaxed with xylene and rehydrated with 100%, 90%, 70%, and 50% ethanol at room temperature. Proteinase K was used to digest the samples, then 4% paraformaldehyde fixed at room temperature for 10 minutes. After hybridization with the double DIG-labeled CCAT1 probe at 55°C overnight, samples were incubated with HRP-conjugated anti-mouse IgG antibody (1:5,000; ISH Detection Kit) at 4°C for 30 minutes. At the end, diaminobenzidine was used to develop the staining. IHC was performed with appropriate primary antibodies. By using the semiquantitative grading system from our previous study (26), each sample was scored independently by two pathologists. Consensus was reached after discussion when scores were not consistent.

**Immunofluorescence**

Nuclear and cytosolic fraction separation was performed using a PARIS Kit (AM1921; Life Technologies) and RNA probe with the sequence 5′-ACCATGCGACCGCGTCCA GTTACCAGGTTCCTCT TTATGGCTAACGCTT-3′ was designed and synthesized by Ruibo according to the manufacturer’s instructions. Briefly, cells seeded on glass coverslips in 6-well plates were fixed with 4% paraformaldehyde for 10 minutes, then permeabilized with 0.1% Triton X-100–PBS for 5 minutes. Five percent BSA-PBS was used to block cells for 1 hour at room temperature and cells were incubated with primary antibody at 4°C overnight, followed by incubation with fluorescent dye–conjugated secondary antibody (Invitrogen) for 1 hour, and then stained with DAPI. Finally, the fluorescence of cells was analyzed by a fluorescence confocal microscope.

**Statistical analysis**

CCAT1 expression data from patients were downloaded from TCGA (http://cancergenome.nih.gov) and the MSKCC (www.mskcc.org) database. All of the statistical analyses were performed using the SPSS 16.0 software version 16.0. All of the experiments listed above were repeated three times and a two-tailed Student t test was utilized for statistical analyses. Data are presented as means and SD. Statistical significance was set as “P < 0.05, **P < 0.01, and ***P < 0.001.

**Results**

CCAT1 is upregulated in AR-negative cells and CRPC tissues and is associated with a poor prognosis

According to the expression of CCAT1 in different stage of prostate cancer tissues, we took functional and mechanism assays to investigate the biological function in the progression of CRPC (Fig. 1A). First, to investigate the expression of CCAT1 in different
CCAT1 expression in CRPC cell and ADPC cells

A

Re-analysis of TCGA, MSKCC database and Tissues verification

Distribution in cell nucleus and cytoplasm

Function in CRPC cell and ADPC cells

Mechanism in cell nucleus and cytoplasm

B

15
10
5
0

CCAT1/GAPDH expression

PC3 DU145 LNCaP

D1

D2

D3

E

1.0
0.1
0.01
0.001

Log2 (CCAT1 RPKM)

TCGA PRAD

P<0.008

n = 29

n = 131

n = 19

G

MSKCC

n = 32

n = 83

n = 41

n = 93

H

Overall survival

Low level of CCAT1

High level of CCAT1

Biochemical relapse-free survival

Figure 1.

CCAT1 is upregulated in AR negative cells and CRPC tissues, and is associated with a poor prognosis. A, Experimental flowchart. B, The expression of CCAT1 was significantly upregulated in CRPC cell lines (PC3 and DU145) relative to ADPC cell lines (LNCaP; P < 0.05). C, After nuclear and cytosolic separation, RNA expression levels were measured by qRT-PCR. GAPDH was used as a cytosolic marker and U6 was used as a nuclear marker. CCAT1 was localized differently in different cell lines. D1, ISH staining of ADPC and CRPC tissues with a CCAT1 probe showed that CCAT1 expression was higher in tumor tissues compared with the normal tissues adjacent to the tumor. D2, CRPC tissues showed higher CCAT1 expression compared with ADPC tissues. D3, Metastatic lymph nodes showed higher CCAT1 expression compared with ADPC tissues. E, TCGA database showed that CCAT1 expression was significantly upregulated in tumors compared with normal tissues (P = 0.008). F, The MSKCC database showed that CCAT1 expression was significantly underexpressed in normal tissues (P = 0.004) compared with PCA tissues and was significantly upexpressed (P < 0.001) in MET tissues compared with the PCA tissues. G, Kaplan–Meier analysis of biochemical relapse-free survival for patients who underwent radical prostatectomy, with a follow-up longer than 12 months (data acquired from MSKCC). Patients with high CCAT1 expression exhibited a lower survival rate than those with low CCAT1 expression (P = 0.0146). H, Kaplan–Meier analysis of biochemical relapse-free survival for patients (data acquired from MSKCC). The overall survival in months for patients with high CCAT1 expression was much shorter compared with patients with low levels of CCAT1 (P = 0.033).
prostate cancer cell lines, we performed qRT-PCR in PC3, Du145, and LNCaP cells and found that CCAT1 expression levels were increased significantly in CRPC cells (PC3 and Du145) relative to ADPC cells (LNCaP; Fig. 1B). Next, we performed a subcellular fractionation location assay (Fig. 1C) and immunofluorescence staining (Supplementary Fig. S1) to investigate CCAT1 location. The results suggested CCAT1 located differently in different cell lines. The cytoplasm location for CCAT1 mainly existed in Du145 cell line and in LNCaP cell line, we could observe CCAT1 location both in cytoplasm and nucleus. Consistent with the above results, ISH results revealed that CCAT1 expression levels were increased in tumor tissues compared with normal tissues adjacent to the tumor (Fig. 1D1). In CRPC tissues and metastatic lymph nodes (Fig. 1D2 and D3), we also observed higher levels of CCAT1 expression. To validate the CCAT1 expression profile in prostate cancer, the largest TCGA-prostate adenocarcinoma database was investigated and CCAT1 expression was found to be significantly upregulated in tumors compared with normal tissues (Fig. 1E, \( P = 0.008 \)). By analyzing the MSKCC database, which includes 29 normal adjacent tissues, 131 primary prostate cancer tissues, and 19 metastatic prostate cancers (MET; Fig. 1F), CCAT1 expression was significantly overexpressed in prostate cancer tissues compared with normal tissues (\( P = 0.004 \)); whereas expression was significantly upregulated (\( P < 0.001 \)) in MET tissues compared with the prostate cancer tissues. Finally, Kaplan–Meier analysis using the log-rank test shows that among those patients who underwent radical prostatectomy with a follow-up longer than 12 months, the biochemical relapse-free survival in those patients with low levels of CCAT1 was significantly longer than that in patients with high levels of CCAT1 (\( P = 0.0146 \); Fig. 1C). For patients with high levels of CCAT1, the overall survival in months was much shorter compared with patients with low levels of CCAT1 (Fig. 1H, \( P = 0.033 \)). Compared with the primary lesion of prostate cancer, expression of CCAT1 in different metastatic sites was also significantly higher (TCGA database; Supplementary Fig. S2, \( P < 0.001 \)).

All results from the MSKCC database suggested that CCAT1 represents a poor prognostic factor for patients with CRPC.

CCAT1 promotes prostate cancer cells’ proliferation, colony formation, and cell cycle and suppresses apoptosis in vitro

To elucidate the mechanism of CCAT1 function, we assessed the effect of CCAT1 on several biologic properties of prostate cancer cells in vitro. First, as shown in Fig. 2A, plasmid-mediated overexpression was used for exogenously manipulating the expression of CCAT1 in LNCaP cells. Then, we examined cellular proliferation by CCK-8 and colony formation assays, cell cycle by PI, and cell apoptosis by Annexin V-FITC and PI. Finally, the proteins related to cell cycle and apoptosis were analyzed by Western blotting. Compared with empty control plasmid, we observed that in LNCaP cells (AR-positive cells), overexpression of CCAT1 not only promoted cellular growth (Fig. 2B), colony formation (Fig. 2C1 and C2), cell-cycle progression (the percentage of cells in G2 increased from 17.19% to 32.64%; Fig. 2D1, D2, and E); but also dramatically decreased cellular apoptosis from 5.73% to 2.46% (Fig. 2F and G). Western blotting analysis revealed that overexpression of CCAT1 also reduced P53 (apoptosis-related) and P21 (cell-cycle-related) protein levels (Fig. 2H; Supplementary Fig. S6). When we used Lv-anti-CCAT1–mediated knockdown to interfere with CCAT1 expression in PC3 cells (AR-negative cells; Fig. 2I), we observed that compared with Lv-NC, knockdown of CCAT1 not only inhibited the cell growth (Fig. 2J), colony formation (Fig. 2K1 and K2), and the cell-cycle progression (the percentage of cells in G2 decreased from 30.19% to 11.85%; Fig. 2L1, L2, and M); it also dramatically increased cellular apoptosis from 5.67% to 11.2% (Fig. 2N and O). Western blotting analysis showed a reduction in CCAT1 increased CASPASE3 (apoptosis-related) and P21 (cell-cycle-related) protein levels (Fig. 2P; Supplementary Fig. S6). Similar results were also observed in another AR-negative cell line, that is, Du145 cells (Supplementary Figs. S3 and S6). Moreover, GSEA showed that a negatively enriched expression of gene sets was involved in DNA repair and Gene Ontology annotation suggests the significant changes in P53 signaling pathways (Fig. 2Q and R) in CCAT1 knockdown prostate cancer cells. Collectively, these results demonstrated that CCAT1 promotes prostate cancer cell growth by enhancing cellular proliferation, cell-cycle progression, while reducing apoptosis.

Reduction in CCAT1 suppresses the formation of prostate xenograft tumors in vivo

To determine whether CCAT1 possesses tumor-promoting effects in prostate cancer, xenograft tumor experiments were performed in nude mice by monitoring tumor latency, incidence, and endpoint weights. We first generated stable CCAT1 knockdown cell line by transcfecting PC3 cells with a Lv-anti-CCAT1 vector to downregulate CCAT1 expression. GFP control vector was utilized as negative control (Fig. 3A and B). Then, we implanted these infected PC3 cells into nude mice. As shown in Fig. 3C, D, F, and G, silencing CCAT1 by lentivirus dramatically suppressed tumor growth as measured by tumor size and weight. Next, we performed hematoxylin and eosin (H&E) and IHC staining of Ki67, activated CASPASE3 in the endpoint tumors (Fig. 3E). The results revealed that significantly reduced Ki67-positive cells and increased CASPASE3–positive cells in CCAT1 knockdown PC3 tumors. These data demonstrated that reduced CCAT1 expression inhibits prostate tumor regeneration and growth by suppression of proliferation and promotion of apoptosis.

Altogether, the above experiments further confirmed that CCAT1 serves as an oncogenic factor in tumorigenesis of prostate cancer.

CCAT1 promotes prostate cancer cell proliferation and colony formation by competing for MIR-28-5P in cytoplasm

Previous studies have suggested that crosstalk between lncRNAs and mRNAs occurs by competing for miRNA response elements. We hypothesized that CCAT1 regulates prostate cancer cell proliferation and colony formation in the same way. Therefore, we carried out RNA pull-down assays and RNA-seq to uncover the miRNA with which CCAT1 competes to regulate biologic behavior in prostate cancer cells. As shown in Fig. 4A and B and Supplementary Table S2, MIR-28-5P, along with MIR-181a-5P, MIR-130a-3P, MIR-130b-3P, MIR-125a-3P, and MIR-125b-5P can interact with CCAT1 (the expression level of CCAT1 was tested by qRT-PCR after prostate cancer cells were transfected with miRNA mimics). Among these miRNAs, we showed that MIR-28-5P was the most sensitive RNA to bind to CCAT1 (Fig. 4B; Supplementary Fig. S4). We next identified the expression of MIR-28-5P in different prostate cancer cells and explored its tumor-suppressing effect. As shown in Fig. 4C–E, MIR-28-5P showed higher expression in Du145 cells compared...
You et al.

CCAT1 promotes prostate cancer cell proliferation, colony formation, and cell-cycle kinetics; and suppresses apoptosis in vitro. A, Plasmid-mediated CCAT1 overexpression in LNCaP cells. B, CCK8 assay showed that CCAT1 overexpression promoted growth rate in LNCaP cells. C1 and C2, Overexpression of CCAT1 promoted colony formation in LNCaP cells. D1, D2, and E, Overexpression of CCAT1 promoted the progression of cell cycle in LNCaP cells. F and G, Overexpression of CCAT1 decreased cellular apoptosis in LNCaP cells. H, Western immunoblotting analysis revealed that overexpression of CCAT1 reduced P53 (apoptosis-related) and P21 (cell-cycle-related) protein level. I, Lv-anti-CCAT1-mediated CCAT1 knockdown in PC3 cells. J, CCK8 assay showed that CCAT1 knockdown inhibited growth rate in PC3 cells. K1 and K2, Knockdown of CCAT1 inhibited colony formation in PC3 cells. L1, L2, and M, Knockdown of CCAT1 inhibited the progression of cell cycle in PC3 cells. N and O, Knockdown of CCAT1 increased cellular apoptosis in PC3 cells. P, Western blotting analysis showed that reduction of CCAT1 increased caspase-3 (apoptosis-related) and P21 (cell-cycle-related) protein levels. Q, GSEA showed that a negatively enriched expression of gene sets was involved in DNA repair in CCAT1 knockdown prostate cancer cells. R, Gene Ontology annotation indicated that significant changes have been made in P53 signaling pathways in CCAT1 knockdown prostate cancer cells.

with other prostate cancer cells, and repressed proliferation and colony formation of Du145 cells. Moreover, we performed antagonist effects experiments by cotransfecting Du145 cells with a Lv-anti-CCAT1 vector and MIR-28-5P inhibitor. Results revealed that the inhibitory action of the Lv-anti-CCAT1 vector on proliferation and colony formation could be partially reversed by the MIR-28-5P inhibitor (Fig. 4F and G). Next, we used qRT-PCR to quantify the expression levels of MIR-28-5P and CCAT1 after various interventions (CCAT1 knockdown or transfection with MIR-28-5P mimics, or MIR-28-5P inhibitor). The results showed that treatment with Lv-anti-CCAT1 vector, MIR-28-5P mimics, or MIR-28-5P inhibitor in Du145 cells,
Figure 3.
Reduction in CCAT1 suppresses the formation of prostate xenograft tumors in vivo. **A**, Fluorescence microscopy was used for the detection of transfection efficiency for LV-anti-CCAT1 transfection, and the results showed that efficiencies were all greater than 90%. **B**, Expression of CCAT1 was measured by qRT-PCR in PC3 cells transfected with LV-anti-CCAT1 vector, which were then implanted into nude mice. **C** and **D**, Subcutaneous tumors that formed in nude mice using stably transfected PC3 cells showed inhibition of CCAT1 or controls at 20 days (n = 5/group). **E**, H&E and IHC staining of Ki67, activated CASPASE-3 in the endpoint tumors revealed that significantly reduced Ki67-positive cells and increased CASPASE-3-positive cells in CCAT1 downregulated PC3 tumors. Scale bars represent 50 and 100 μm. Each bar represents the mean ± SD of three independent experiments. **F**, P < 0.05. **G**, Tumor formation growth curves after transfection with indicated cells. **G**, Histograms describing the mean tumor weights of each group. Mean tumor volumes are plotted.
CCAT1 promotes prostate cancer cell proliferation and colony formation by competing for MIR-28-5P in cytoplasm. A, RNA pulldown assays and RNA-seq showed MIR-181a-5P, MIR-130a-3P, and MIR-130b-3P interacted with CCAT1, and expression levels of CCAT1 were tested using qRT-PCR after prostate cancer cells were transfected with these 3 miRNAs mimics. B, MIR-28-5P was the most sensitive miRNA to bind to CCAT1. C, MIR-28-5P showed higher expression in Du145 cells compared with other prostate cancer cells. D1, D2, and E, MIR-28-5P manifested an anticancer effect in Du145 cells. F and G, The inhibitory effect of Lc-anti-CCAT1 vector on proliferation and colony formation was partially reversed by MIR-28-5P inhibitor. H, I, and J, qRT-PCR revealed that treatment with Lc-anti-CCAT1 vector, MIR-28-5P mimics, or MIR-28-5P inhibitor in Du145 cells upregulated the expression of MIR-28-5P, downregulated the expression of CCAT1, and upregulated the expression of CCAT1 respectively. J and K, Dual luciferase reporter assays demonstrated that MIR-28-5P significantly reduced luciferase activity (wild-type), and mutation of the MIR-28-5P-binding site in CCAT1 abrogated the inhibitory effects.

The above results illustrated that CCAT1 can compete with MIR-28-5P to promote malignant biologic behavior in prostate cancer cells.

CCAT1 plays a role in posttranscriptional regulation of AR-targeted genes, thus promoting progression of prostate cancer

As described above, Clark and colleagues (17, 25) had identified a role for DDX5 (P68) in the posttranscriptional regulation of AR-mediated genes. In our RNA pulldown assay, P68 was detected to interact with CCAT1 (Fig. 5A–C). To investigate whether CCAT1 functions in the progression of prostate cancer by interacting with P68, we first utilized the RIP assay to verify the interaction between CCAT1 and P68. It showed that CCAT1 was dramatically enriched in the P68-immunoprecipitation compared with control IgG (Fig. 5D). Next, to identify whether CCAT1 regulates the AR-mediated gene expression, CHIP assays were conducted to measure the enrichment levels of the AR-mediated gene PSA (Exon 3, Exon 5, Introns 3 fragments) in CCAT1 knockdown and overexpressing LNCaP cells. We then used qRT-PCR and Western blotting analyses to examine PSA expression at both the mRNA and protein levels after LNCaP cells being treated with Lc-anti-CCAT1 vector or overexpressing vector. As shown in Fig. 5E and F and Supplementary Fig. S6, knockdown of CCAT1 decreased the enrichment levels of AR-mediated gene (PSA) fragments and PSA expression at both the mRNA and protein levels in Fig. 5E and F and Supplementary Fig. S6, knockdown of CCAT1 decreased the enrichment levels of AR-mediated gene (PSA) fragments and PSA expression at both the mRNA and protein levels (HNF3X.1 was also a downstream gene of AR signaling pathway). In addition, CCK8, cell colony formation and apoptosis assays were performed in LNCaP cell to investigate whether the tumor-promoting effects of
CCAT1 promotes prostate cancer cell proliferation

CCAT1 would be abolished after knockdown P68. As shown in Fig. 5G and J, and Supplementary Fig. S6, downregulation of P68 in CCAT1 overexpression cell could dramatically reduce the tumor-promoting effect of CCAT1. Besides AR-regulated prodifferentiation gene PSA, we also examined the expression level of AR-regulated stemness and castration resistance gene, UBE2C, after CCAT1 overexpression. As shown in Supplementary Fig. S5, CCAT1 upregulation could increase AR-mediated castration resistance gene UBE2C’s expression, promoting CRPC progression.

Taken together, these results suggest that CCAT1 promotes AR-mediated genes expression by binding to P68 and functions as an oncogene in the progression of prostate cancer (Fig. 5K).

Discussion

Numerous newly discovered lncRNAs have recently been proven to play important roles in human diseases, especially in cancer. In this study, we provided convincing evidence to show that CCAT1 behaves as an oncogenic factor in the progression of prostate cancer, especially in the transition from ADPC to CRPC. The level of CCAT1 expression in prostate cancer tissues was significantly higher than in corresponding nontumorous tissues. More importantly, in CRPC cells, CRPC tissues, and metastatic lymph nodes, its expression was significantly higher than in ADPC cells and tissues. Furthermore, the expression of CCAT1 was inversely related to the survival of patients with CRPC. From the experiments performed in vitro and in vivo, we observed that CCAT1 promoted prostate cancer cell proliferation, colony formation, as well as cell-cycle progression, while repressed apoptosis. Notably, we identified a phenomenon of CCAT1 “sponging” MIR-28-5P to reverse the anticancer effect in cytoplasm and serves as a scaffold for P68 and the AR transcriptional complex, which regulates AR-mediated genes expression in the nucleus. Consequently, CCAT1 promotes the CRPC progression.

Investigators have in recent years identified a series of cancers associated with the upregulation of CCAT1. It has been shown that CCAT1 can “sponge” MIR-143 in thyroid carcinoma to

![Image](https://www.aacrjournals.org/molcanther/article-pdf/18/12/2477/538974/mct-19-0095.pdf)
activate the PI3K/AKT and MAPK signaling pathways (27), as well as in non–small cell lung cancer, CCAT1 stimulates the elements of the Wnt signaling pathway by blocking Let-7c function (28). Similarly, the CCAT1/MIR-181a-5P/HOXA1 axis was shown to exert an oncogenic role in multiple myeloma (29). Although the biologic function of CCAT1 in different types of cancers has been reported, a role in the development of prostate cancer has not yet been illustrated. We demonstrated that in Du145, CCAT1 plays its oncogenic role by “sponging” MIR-28-5P to boost prostate cancer cell proliferation. We chose Du145 cells to study this regulatory mechanism for the following reasons: (i) CCAT1 exhibits higher expression in Du145 cell cytoplasm and MIR-28-5P showed higher expression in Du145 cells compared with PC3 or LNCaP cells, (ii) Scholars (2, 30) have pointed out that lncRNAs can transactivate mRNA’s decay by duplexing with 3’ cells; (iii) CCAT1 exhibits higher oncogenic role by “sponging” MIR-28-5P to boost prostate cancer proliferation, and (iv) P68 has also been hypothesized that there might be a similar mechanism at work related to the oncogenic role of CCAT1 in prostate cancer. P68 is a developmentally regulated prototypical member of the DEAD box enzyme family, which has been reported to perform important roles in many cellular processes affecting cancer progression including miRNAs, pre-mRNA processing (33, 34), cellular proliferation, and ribosomal biogenesis (35). P68 has also been identified as an AR-interacting protein and is associated with the promoter region of AR-responsive genes. In our pulldown assays, CCAT1 sense and antisense strands were transcribed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In summary, CCAT1 boosted prostate cancer cell proliferation by competing for MIR-28-5P in cytoplasm and interacting with P68 to activate AR-mediated genes (PSA, UBE2C) in the nucleus, promoting CRPC progression. Our study revealed that CCAT1 displays different regulatory strategies in the malignant progression of prostate cancer and may supply a strategy for targeting CCAT1 as a potential biomarker and a corresponding therapeutic target for patients with CRPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. You, B. Xu, M. Chen
Development of methodology: Z. You, Ya Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. You, C. Liu, C. Wang, Yi. Wang, S. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. You, Z. Ling, Yi. Wang, M. Zhang
Writing, review, and/or revision of the manuscript: Z. You, C. Liu, B. Xu, H. Guan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. You, C. Liu
Other (collection of the pathologic section): M. Zhang

Acknowledgments
This study was funded by the National Natural Science Foundation of China (nos. 81872089, 81770849, 81672551,81300472, 81070592, 81202268, 81202034), Natural Science Foundation of Jiangsu Province (BK 20150642, 20161434), and National Science Foundation of Anhui Province (K2018A0214).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 7, 2019, revised June 11, 2019; accepted July 29, 2019; published first August 6, 2019.

References


LncRNA CCAT1 Promotes Prostate Cancer Cell Proliferation by Interacting with DDX5 and MIR-28-5P

Zonghao You, Chunhui Liu, Can Wang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-19-0095

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2019/08/06/1535-7163.MCT-19-0095.DC1

Cited articles
This article cites 33 articles, 7 of which you can access for free at:
http://mct.aacrjournals.org/content/18/12/2469.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link:
http://mct.aacrjournals.org/content/18/12/2469.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.