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LINC00675 suppresses proliferative, migration and invasion of clear cell renal cell carcinoma *via* the Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: To clarify the role of LINC00675 in affecting the progression of clear cell renal cell carcinoma (ccRCC) and its potential mechanism, thus providing effective hallmarks and therapeutic targets for the clinical treatment of ccRCC.

MATERIALS AND METHODS: Differentially expressed long non-coding RNAs (lncRNAs) in renal epithelial tissues and ccRCC tissues were searched by analyzing the dataset downloaded from The Cancer Genome Atlas (TCGA) and LINC00675 was selected. LINC00675 level in ccRCC cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Overexpression model of LINC00675 model in 786-O and 769-P cells was constructed by the transfection of pcDNA3.1(+)-LINC00675 (LV-LINC00675). Changes in proliferative, migratory, and invasive capacities of 786-O and 769-P cells overexpressing LINC00675 were assessed. At last, relative levels of β-catenin, Vimentin, and N-cadherin in ccRCC cells overexpressing LINC00675 were detected by qRT-PCR and Western blot.

RESULTS: LINC00675 was downregulated in ccRCC tissues and cell lines. Overexpression of LINC00675 attenuated proliferative, migratory, and invasive capacities of 786-O and 769-P cells. Downregulation in β-catenin after overexpression of LINC00675, while Vimentin and N-cadherin levels did not change.

CONCLUSIONS: LINC00675 is downregulated in ccRCC. Overexpression of LINC00675 attenuates ccRCC to proliferate, migrate, and invade by activating the Wnt/β-catenin pathway.

Key Words:
CcRCC, LINC00675, Wnt/β-catenin.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors in the urinary system[1]. The incidence of ccRCC accounts for 5% of adult malignancies, presenting an increased trend globally[2]. Clear cell RCC (ccRCC) is the most prevalent subtype of RCC, accounting for 70-75% of all RCC cases[3]. About 20-30% of ccRCC patients are accompanied by distant metastasis at the initial diagnosis owing to the ineffective early-stage diagnostic methods[4]. Surgery is the gold standard for localized ccRCC. Nevertheless, nearly 20% localized ccRCC patients suffer from postoperative recurrence. Chemotherapy and radiotherapy for ccRCC are insensitive. Once lymphatic metastasis occurs, the overall survival of ccRCC patients is shorter than 5 years even undergoing radical lymph node dissection[5]. It is necessary and urgent to uncover new tumor hallmarks and therapeutic targets for ccRCC.

Long non-coding RNA (lncRNA) is a transcript with over 200 bases long and does not have protein-coding potential[6,7]. With the completion of the Human Genome Project, protein-encoding RNAs only account for 3% of all genomes and the remaining are non-coding RNAs. LncRNAs are the majority of non-coding RNAs, which were initially considered as sequence garbage without any biological functions[8,9]. In recent years, increasing evidence has proved the significant roles of lncRNAs in the biological progressions. According to the relative positions on the genome with protein-coding genes, lncRNAs are classified into antisense lncRNAs, intronic transcripts, large intergenic non-coding RNAs, promoter-associated lncRNAs, and UTR-associated lncRNAs. The major functions of lncRNAs in human diseases include: (1) LncRNAs induce transcription of the protein-encoding genes in the upstream promoter regions, thereby interfering with expressions of downstream specific genes; (2) LncRNAs mediate chromatin remod-

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eling and histone modification, or inhibit RNA polymerase II, thereby affecting the expressions of downstream genes; (3) LncRNAs contribute to form a complementary double-strand with protein-encoding genes, further forming endogenous small interfering RNAs to regulate gene expressions; (4) LncRNAs change subcellular distribution of proteins by binding to them or regulating protein activities; (5) LncRNAs could serve as RNA precursors; (6) LncRNAs contribute to form a nucleic acid-protein complex together with proteins[10]. The vital functions of lncRNAs in regulating pathological processes have been well studied[11,12].

LncRNAs exert fundamental effects on tumor progression by influencing tumor epigenetics, signaling pathways, and cross-regulation as skeletal molecules, guide molecules, decoy molecules, and signaling molecules[13-15]. Abnormally expressed lncRNAs are involved in different stages of tumor progressions[16-18]. Tumor metastasis is also closely related to lncRNAs[19,20]. Moreover, diagnostic and prognostic potentials of lncRNAs in tumor diseases emerged as effective, non-invasive methods. In this paper, differentially expressed lncRNAs in renal epithelial tissues and ccRCC tissues were searched by analyzing the dataset downloaded from TCGA and LINC00675 was selected. We aim to uncover the role of LINC00675 in influencing the progression of ccRCC.

Materials and Methods

Data Collection and Processing

GDC TCGA Kidney Clear Cell Carcinoma (KIRC) containing genome-wide expression profile of ccRCC patients was downloaded from UCSC Xena platform (bioRxiv 326470; doi: https://doi.org/10.1101/326470) on atacseq.xenahubs.net. Expression data for LINC00675 expressions in 72 normal controls and 526 ccRCC tissues were obtained by searching for ENSG00000263429.

Cell Culture

RCC cell lines (786-O, CAKI-1, ACHN, and 769-P) and renal tubular epithelial cell line (HK2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and 1% penicillin-streptomycin (HK2 cells in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), 786-O and 769-P cells in Roswell Park Memorial Institute-1640 (RPMI-1640), CAKI-1 cells in McCoy's 5A and ACHN cells in DMEM (HyClone, South Logan, UT, USA).

Lentivirus Transfection

The full-length complementary deoxyribose nucleic acid (cDNA) of human LINC00961 was amplified and cloned into pcDNA3.1 and pcDNA3.1(+)-LINC00675 was obtained. ShLuc was utilized to construct negative control sequences. Cells were transfected with LV-LINC00675 or LV-NC and transfection-positive cells were screened by applying 800 μg/mL G418.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cells were lysed to harvest RNAs using TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNAs was subjected to reverse transcription according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates with 6.0×10^3 cells per well. At the established time

Table I. Primers for real-time PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	TATCGGACGCCTGGTTAC	TATCGGACGCCTGGTTAC
LINC00675	GCCTACTGCTCTGGATCATCTGGTA	ACCTGCGTCTCTTCTCCTCTTCC
N-cadherin	GACAATGCCCCTCAAGTGTT	CCATTAAGCCGAGTGATGGT
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
β-catenin	TGGCATCAAGGGAGACAC	TGACCTAACTAAAGCACCAGA

points, 10 μL of cell counting kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells were inoculated into 96-well plates with 6×10^3 cells per well and labeled with 100 μL of EdU reagent (50 μM) per well for 2 h (Thermo Fisher Scientific, Waltham, MA, USA). After washing with phosphate-buffered saline (PBS), cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine and permeated with 100 μL of penetrant. After washing with PBS once, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in dark for 30 min. The EdU-positive ratio was determined under a fluorescent microscope (magnification 20×).

Transwell

Cell density was adjusted to 2×10^4 cells/ml. 100 μL of suspension was applied in the upper side of the transwell chamber (Corning, Corning, NY, USA) coated with diluted Matrigel (1:7 diluted in RPMI-1640). In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, cells penetrated to the bottom side were fixed in 4% paraformaldehyde for 20 min, stained with crystal violet for 20 min, and counted using a microscope. The number of penetrating cells was counted in 5 randomly selected fields per sample (magnification 200×).

Western Blot

Cells were lysed for extracting proteins using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 2 h, membranes were subjected to incubation with primary and secondary antibodies. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) were used for data analysis. Data were expressed as mean ± standard deviation ($\overline{x} \pm SD$). Intergroup data were compared using the *t*-test. $p<0.05$ was statistically significant.

Results

Downregulation of LINC00675 in ccRCC

A dataset containing 72 normal tissues and 526 ccRCC tissues was downloaded. LINC00675 level was markedly downregulated in ccRCC tissues (Figure 1A). Among them, we selected 70 matched paracancerous tissues and ccRCC tissues. Likewise, LINC00675 was lowly expressed in ccRCC tissues than adjacent normal ones (Figure 1B). Subsequently, LINC00675 level was found to be lowly expressed in ccRCC cell lines as well (Figure 1C). The above data showed the potential involvement of LINC00675 in the progression of ccRCC.

LINC00675 Overexpression Suppressed Proliferative Capacity of ccRCC

In the following experiments, 786-O and 769-P cells were selected. Transfection of LV-LINC00675 remarkably upregulated LINC00675 level in these two cell lines (Figures 2A, 2B). EdU assay demonstrated lower EdU-positive ratio in 786-O and 769-P cells overexpressing LINC00675 than those of controls (Figures 2C, 2D). Moreover, viabilities in ccRCC cells transfected with LV-LINC00675 were reduced, further verifying the suppressed proliferative capacity (Figures 2E, 2F). Hence, the overexpression of LINC00675 attenuated ccRCC to proliferate.

LINC00675 Overexpression Suppressed Migratory and Invasive Capacities of CcRCC

Early-stage metastasis of ccRCC markedly threats human health and lives. Here, transwell assay showed that transfection of LV-LINC00675 decreased migratory cell number in both 786-O and 769-P cells (Figures 3A, 3B). In addition, invasiveness in ccRCC was attenuated by the overexpression of LINC00675 (Figures 3C, 3D). It is believed that overexpression of LINC00675 suppressed the metastatic ability of ccRCC.

LINC00675 Regulated the Wnt/β-Catenin Pathway in ccRCC

It is reported that EMT and Wnt pathways are closely related to tumor cell proliferation and metastasis[21,22]. Our experiments further examined relative levels of key genes in EMT and Wnt pathways, β-catenin, Vimentin, and N-cadherin

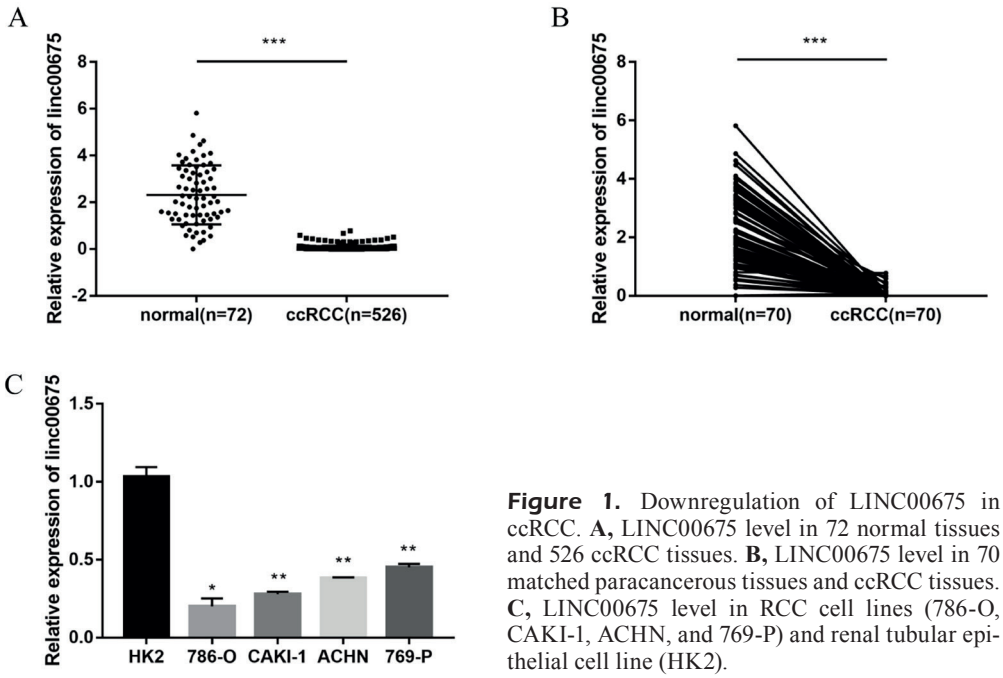

Figure 1. Downregulation of LINC00675 in ccRCC. **A,** LINC00675 level in 72 normal tissues and 526 ccRCC tissues. **B,** LINC00675 level in 70 matched paracancerous tissues and ccRCC tissues. **C,** LINC00675 level in RCC cell lines (786-O, CAKI-1, ACHN, and 769-P) and renal tubular epithelial cell line (HK2).

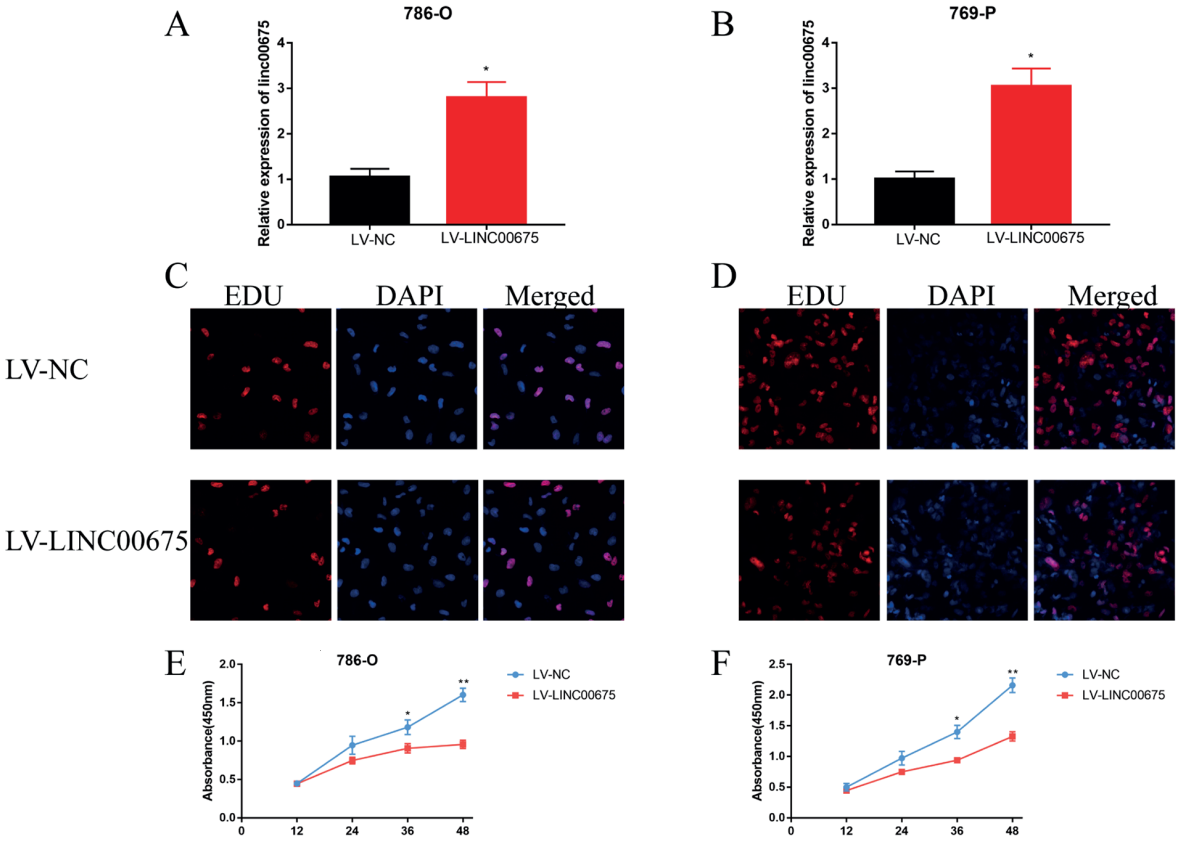

Figure 2. LINC00675 overexpression suppressed proliferative capacity of ccRCC. **A,** Transfection efficacy of LV-LINC00675 in 786-O cells. **B,** Transfection efficacy of LV-LINC00675 in 769-P cells. **C,** EdU-labeled, DAPI-labeled and merged images in 786-O cells transfected with LV-NC or LV-LINC00675 (magnification: 200X). **D,** EdU-labeled, DAPI-labeled and merged images in 769-P cells transfected with LV-NC or LV-LINC00675 (magnification: 200X). **E,** Viability in 786-O cells transfected with LV-NC or LV-LINC00675. **F,** Viability in 769-P cells transfected with LV-NC or LV-LINC00675.

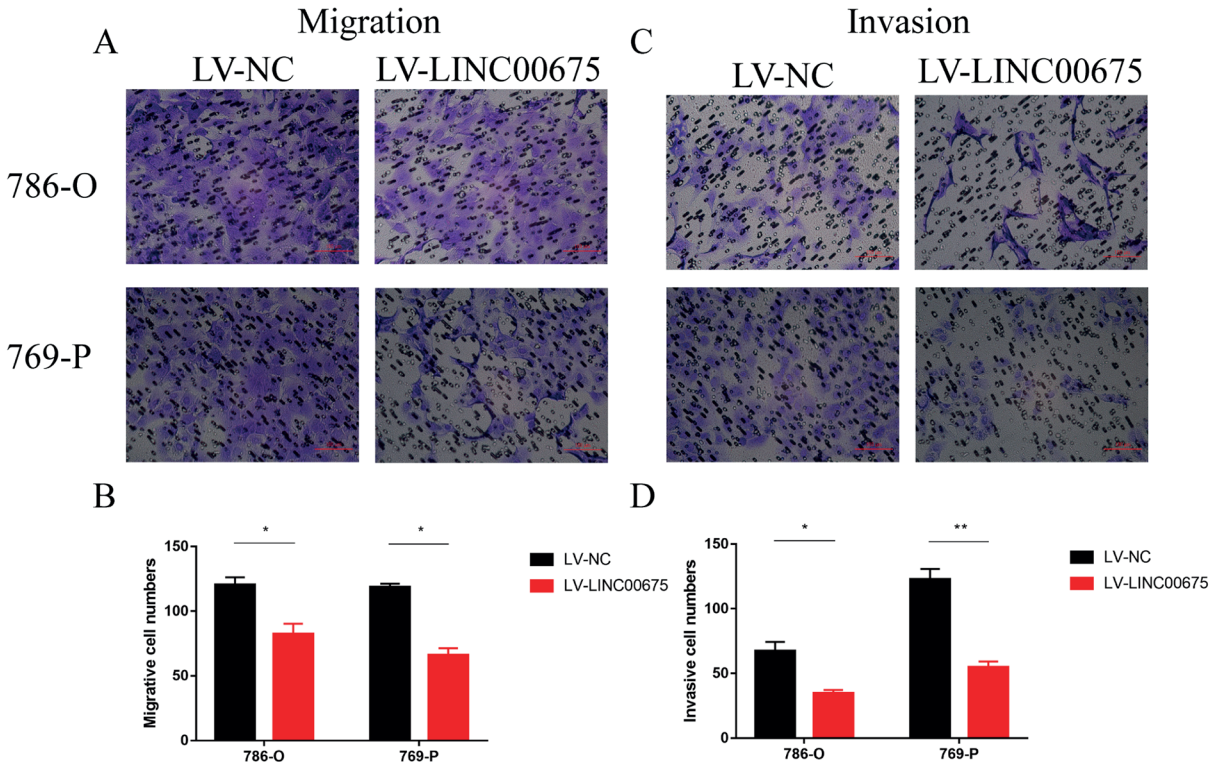

Figure 3. LINC00675 overexpression suppressed migratory and invasive capacities of ccRCC. **A,** Migration in 786-O and 769-P cells transfected with LV-NC or LV-LINC00675 (magnification: 200X). **B,** Migratory cell numbers in 786-O and 769-P cells transfected with LV-NC or LV-LINC00675. **C,** Invasion in 786-O and 769-P cells transfected with LV-NC or LV-LINC00675 (magnification: 200X). **D,** Invasive cell numbers in 786-O and 769-P cells transfected with LV-NC or LV-LINC00675.

in ccRCC cells influenced by LINC00675. Both mRNA and protein levels of β-catenin were down-regulated in 786-O and 769-P cells overexpressing LINC00675. However, no significant differences in Vimentin and N-cadherin levels were observed between ccRCC cells transfected with LV-NC or LV-LINC00675 (Figure 4A-4D). Collectively, LINC00675 influenced the proliferation of ccRCC through the Wnt pathway, rather than the EMT pathway.

Discussion

LncRNAs are key regulators in tumor diseases. They are of significance in tumor proliferation and metastasis. Abnormally expressed lncRNAs in ccRCC affect tumor cell behaviors through translational, post-transcriptional, and epigenetic regulations. Li et al[23] uncovered that LINC00675 is the biomarker for predicting poor overall survival and recurrence of pancreatic ductal adenocarcinoma. In gastric cancer, LINC00675 suppresses its progression by enhancing phosphorylation of Vimentin on Ser83[24]. LINC00675 influences tumor cell proliferation and metastasis by activating the Wnt pathway in cervical cancer[25]. As a miR-942 sponge, LINC00675 alleviates the progression of colorectal cancer by targeting the Wnt pathway[26].

The Wnt pathway is a highly conserved pathway during biological evolution[27,28]. In normal cells, β-catenin acts as a cytoskeletal protein to form a complex with E-cadherin at the cell membrane, thus maintaining cell adhesion and preventing cell migration. Once GSK3B is inactivated by binding of extracellular Wnt with specific receptor Frizzled on the cell membrane, α-catenin escapes from phosphorylation, and degradation. Then, β-catenin is accumulated in the cytoplasm. A certain concentration of cytoplasmic β-catenin triggers nuclear translocation, where nuclear cyclinD1, c-myc, and other proto-oncogenes are activated. It is proved that the Wnt pathway is activated in multiple types of tumors, and it further inhibits downstream proteins, including axin, GSK-3, and APC. The complex axin/GSK-3/APC stimulates

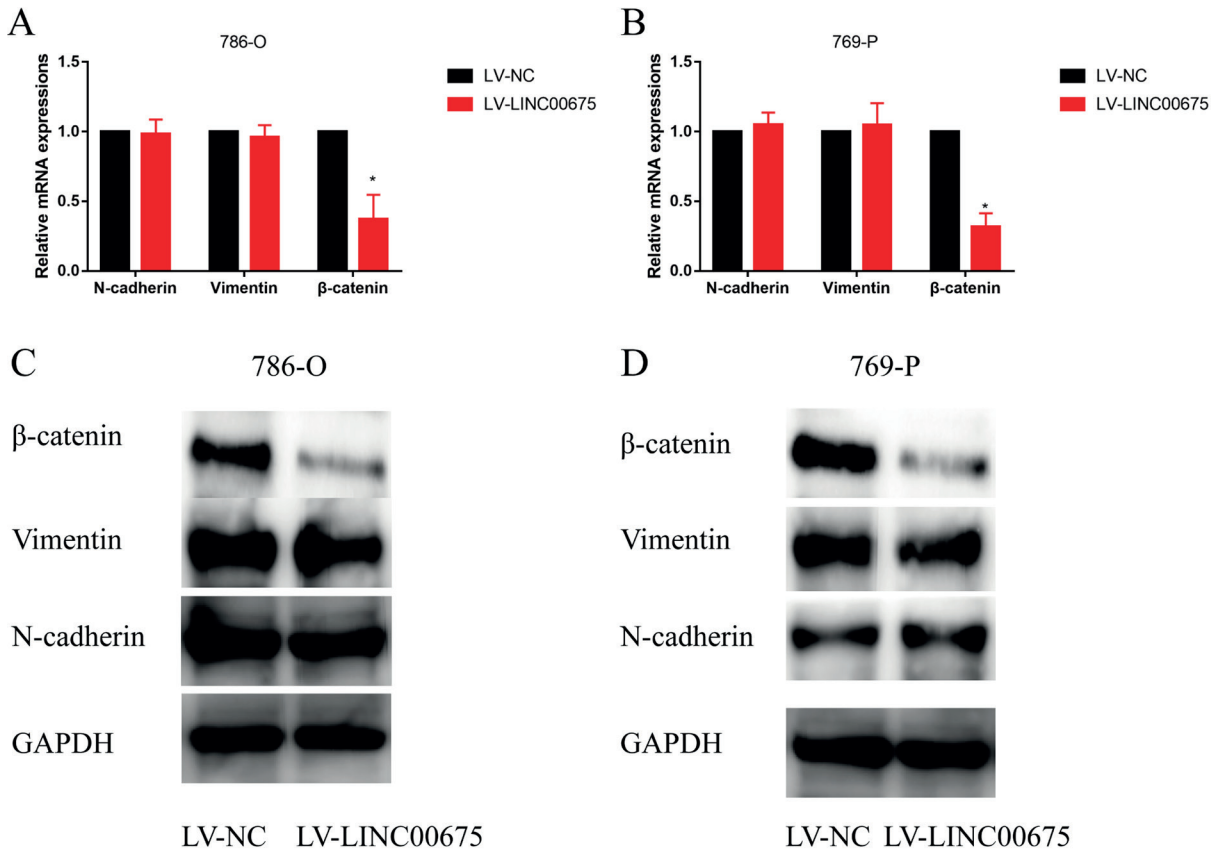

Figure 4. LINC00675 regulated the Wnt/β-catenin pathway in ccRCC. **A,** Relative levels of β-catenin, Vimentin and N-cadherin in 786-O cells transfected with LV-NC or LV-LINC00675. **B,** Relative levels of β-catenin, Vimentin and N-cadherin in 769-P cells transfected with LV-NC or LV-LINC00675. **C,** Protein levels of β-catenin, Vimentin and N-cadherin in 786-O cells transfected with LV-NC or LV-LINC00675. **D,** Protein levels of β-catenin, Vimentin, and N-cadherin in 769-P cells transfected with LV-NC or LV-LINC00675.

the degradation of intracellular β-catenin. Once the degradation of intracellular β-catenin is inhibited, cytoplasmic β-catenin is stably expressed, and partial β-catenin targets on TCF/LEF transcriptional factors to activate downstream genes[21].

Tumor progression is closely related to the Wnt pathway and lncRNAs. LncRNA T-UCR serves as a potential downstream of the Wnt pathway to induce the tumorigenesis of liver and gallbladder[29]. LincRNA CCAL affects the progression of colorectal cancer *via* inhibiting the Wnt pathway[30]. In ccRCC, lncRNA OTUD6B-AS1 predicts poor prognosis[31]. As a ceRNA, LINC01133 sponges miR-106a-3p to inhibit APC level and Wnt pathway in gastric cancer[32]. Our findings uncovered downregulation in β-catenin after the overexpression of LINC00675 in ccRCC. However, the specific mechanism remains to be explored in the future.

Conclusions

Collectively, these data showed that LINC00675 is downregulated in ccRCC. Overexpression of LINC00675 attenuates ccRCC to proliferate, migrate and invade through activating the Wnt/β-catenin pathway. LINC00675 may be utilized as a therapeutic target for ccRCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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