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LINC00707 accelerates the proliferation, migration and invasion of clear cell renal cell carcinoma

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Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) have been well concerned in tumor researches, which are believed to influence tumorigenesis and tumor progression. This study aims to uncover the role of LINC00707 in clear cell renal cell carcinoma (ccRCC) and the underlying mechanism.

MATERIALS AND METHODS: Differentially expressed lncRNAs in ccRCC tissues and renal epithelial tissues were analyzed in The Cancer Genome Atlas (TCGA), and LINC00707 was screened out. Expression level of LINC00707 in ccRCC cell lines was determined as well. Regulatory effects of LINC00707 on influencing proliferative, migratory, and invasive abilities of 786-O and 769-P cells were assessed. At last, relative levels of epithelial-mesenchymal transition (EMT)-related genes E-cadherin and N-cadherin in 786-O and 769-P cells were detected by quantitative real time-polymerase chain reaction (qRT-PCR) and Western blot.

RESULTS: LINC00707 was upregulated in ccRCC tissues and cell lines. Silence of LINC00707 attenuated proliferative, migratory, and invasive abilities of 786-O and 769-P cells. Moreover, knockdown of LINC00707 upregulated E-cadherin and downregulated N-cadherin in ccRCC cells at both mRNA and protein levels.

CONCLUSIONS: LINC00707 is upregulated in ccRCC, which could promote cancer cells to proliferate, migrate, and invade. LINC00707 accelerates the progression of ccRCC by activating EMT pathway.

Key Words:
CcRCC, LINC00707, Proliferation, Metastasis, EMT.

Introduction

Renal cell carcinoma (RCC) is the most common malignancy in the urinary system[1]. Its incidence is on the rise annually, accounting for 5% of all malignancies in adults[2]. Clear cell renal cell carcinoma (ccRCC) is a prevalent subtype of RCC, ranks for 70-75% of all RCC cases[3]. Due to the lack of sensitivity diagnostic methods for early-stage ccRCC, about 20-30% of ccRCC patients are accompanied with distant metastasis at the initial diagnosis[4]. Surgical procedures are the golden standard for the treatment of local RCC. Nevertheless, nearly 20% local ccRCC patients experience postoperative recurrence. Meanwhile, ccRCC is not sensitive to chemotherapy and radiotherapy. Once the occurrence of lymphatic metastasis, the overall survival of metastatic ccRCC is usually within 5 years even performing the radical lymphadenectomy[5]. It is urgent to uncover the pathogenesis of ccRCC, so as to develop effective therapeutic targets for improving the clinical outcomes of ccRCC.

Long noncoding RNA (lncRNA) is a class of RNAs with more than 200 nucleotides that do not have a protein-encoding function. It has a similar structure to that of a mRNA[6,7]. With the advance of the Human Genome Project, about 3% of human genome is identified to be able to encode proteins. The remaining are noncoding RNAs. In the past, lncRNAs were considered to be the by-products of transcription and did not have meaningful biological functions[8]. With the deepening of researches, lncRNAs are found to be closely related to the occurrence and development of various diseases, showing complex biological functions. Currently, lncRNAs are mainly divided into five categories, including antisense lncRNAs, intronic transcripts, large intergenic noncoding RNAs, promoter-associated lncRNAs, and UTR associated lncRNAs. It is reported that lncRNA HOTAIR could suppress gene transcription by binding to the methyltransferase family[9]. LncRNA PXN-AS1-L is resistant to RNA hydrolysis degradation or miRNA sponge by binding to mRNA[10]. LINC00707 is involved in many types of tumors, which is related to disease progno-

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sis[11-13]. This study mainly uncovered the biological role of LINC00707 in ccRCC.

Materials and Methods

Data Acquisition and Analysis

The Cancer Genome Atlas (TCGA) is a very important cancer database, containing clinical data, genomic variations, mRNA expressions, miRNA expressions, methylation, and other data of various types of human cancers. It is an excellent platform for cancer researches. Briefly, 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 LINC00707 expressions in 72 normal controls and 526 ccRCC tissues were obtained by searching for ENSG00000238266.

Cell Culture and Transfection

HK2, 786-O, ACHN, and 769-P cells were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

Cells were pre-seeded in the 6-well plate with 60% confluence. Cell transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 6 h, medium containing 10% FBS was replaced. Transfected cells for 24 h were harvested for other experiments. Sequences of transfection vectors were listed in Table I.

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

Cells were lysed to harvest RNAs using TRIzol method (Invitrogen, Carlsbad, CA, USA), and the extracted RNAs were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). 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) at 94°C for 5 min, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. 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 2.0×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, 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 2×10^3 cells per well and labeled with 100 μL of EdU reagent (50 μM) per well for 2 h (R&D Systems, Minneapolis, MN, 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; Sigma-Aldrich, St. Louis, MO, USA) in the dark for 30 min. EdU-positive ratio was determined under a fluorescent microscope.

Transwell

Cell density was adjusted to 1.5×10^4 cells/mL. 100 μL of suspension was applied in the upper side of transwell chamber (Corning, Corning, NY, USA) coated with Matrigel. In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, cells penetrated

Table I. The list of primers and siRNA sequences.

Gene	Forward	Reverse
LINC00707	TCACATCTGTGAAAAGAGTGCT	CTGGACTGTGAGTACCAGGC
GAPDH	GGGAGCCAAAAGGGTCAT	GAGTCCTTCCACGATACCAA
E-cadherin	GACAACAAGCCCGAATT	GGAAACTCTCTCGGTCCA
N-cadherin	CGGGTAATCCTCCCAAATCA	CTTTATCCCGGCGTTTCATC
siRNA sequences		
si-LINC00707	CAUGACGUGAGAACUUACUAGAGAU	AUCUCUAGUAAGUUCUCACGUCAUG
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

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). Protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membrane (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 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., CA, USA) were used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}$±SD). Intergroup data were compared using the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

Upregulated LINC00707 In CcRCC

A dataset containing LINC00707 expressions in 72 normal controls and 526 ccRCC tissues was downloaded from UCSC Xena in TCGA. LINC00707 was found to be remarkably upregulated in ccRCC tissues relative to normal ones (Figure 1A). After screening the data, a total of 70 paired ccRCC tissues and matched paracancerous tissues were harvested. Identically, LINC00707 was highly expressed in ccRCC tissues relative to controls (Figure 1B). *In vitro* level of LINC00707 remained higher in RCC cells relative to renal tubular epithelial cells (Figure 1C). To further uncover the biological role of LINC00707, we first constructed si-LINC00707 and tested its transfection efficacy. LINC00707 was markedly downregulated by transfection of si-LINC00707 in 786-O and 769-P cells (Figure 1D, 1E). The

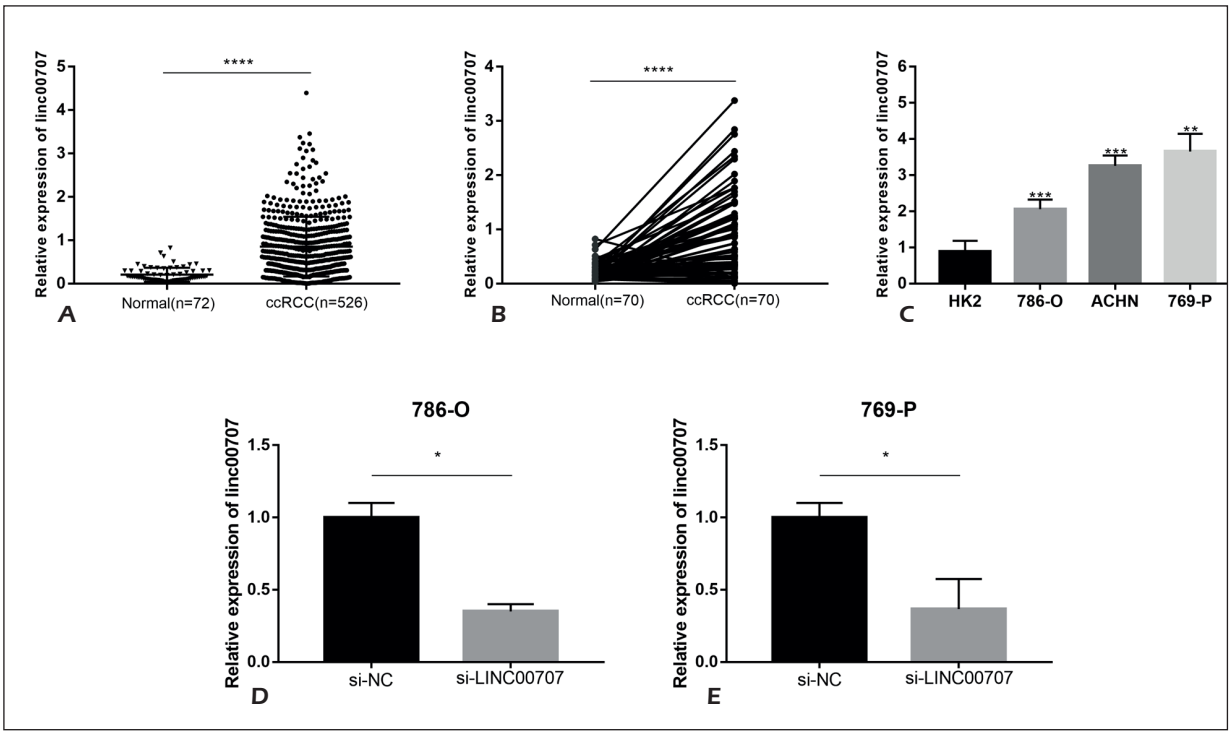

Figure 1. Upregulated LINC00707 in ccRCC. **A**, LINC00707 expressions in 72 normal controls and 526 ccRCC tissues downloaded from UCSC Xena in TCGA. **B**, Relative level of LINC00707 in paired ccRCC tissues and matched paracancerous tissues. **C**, Relative level of LINC00707 in HK2, 786-O, ACHN and 769-P cells. **D**, Transfection efficacy of si-LINC00707 in 786-O cells. **E**, Transfection efficacy of si-LINC00707 in 769-P cells.

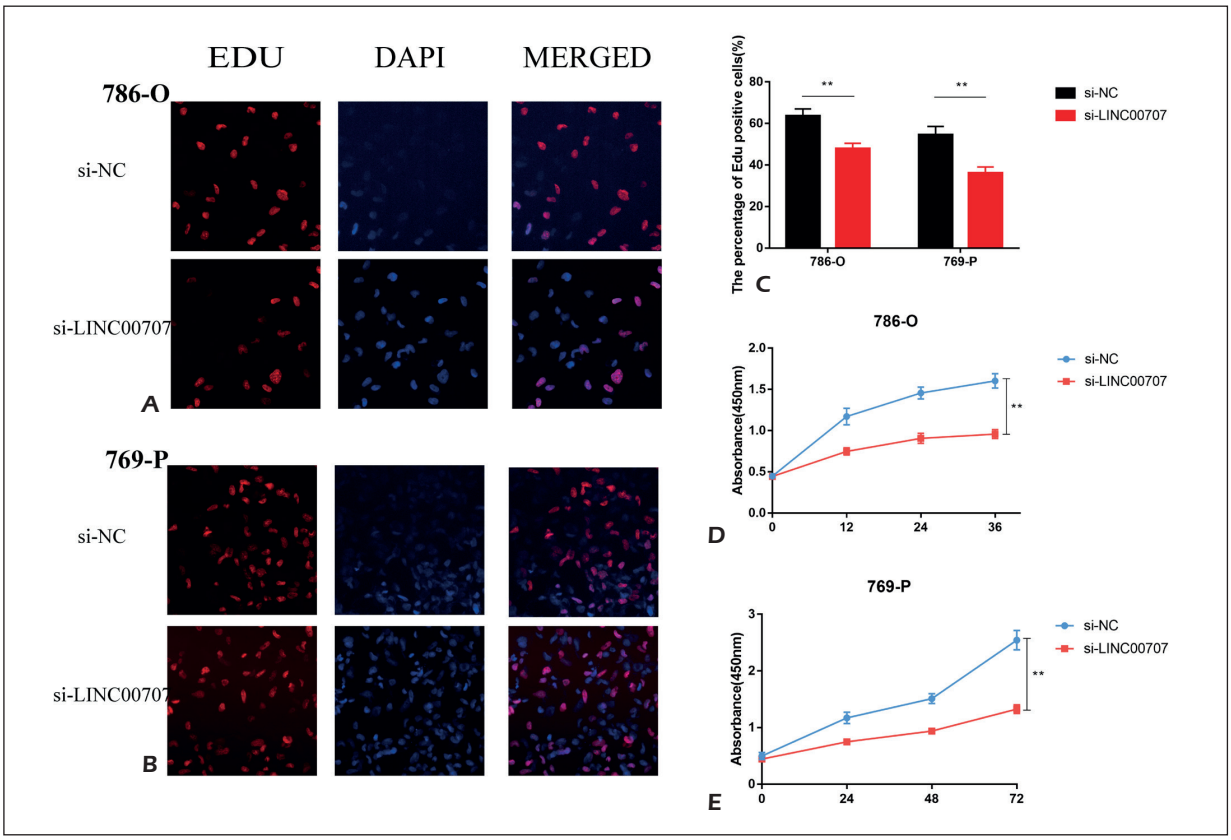

Figure 2. Knockdown of LINC00707 suppressed the proliferative ability of ccRCC. **A**, EdU-labeled, DAPI-stained and merged images of 786-O cells transfected with si-NC or si-LINC00707 (magnification: 400×). **B**, EdU-labeled, DAPI-stained and merged images of 769-P cells transfected with si-NC or si-LINC00707 (magnification: 400×). **C**, Percentage of EdU-positive cells in 786-O and 769-P cells transfected with si-NC or si-LINC00707. **D**, Viability in 786-O cells transfected with si-NC or si-LINC00707. **E**, Viability in 769-P cells transfected with si-NC or si-LINC00707.

above data suggested that LINC00707 may exert a crucial role in the progression of ccRCC.

Knockdown of LINC00707 Suppressed the Proliferative Ability of CcRCC

After transfection of si-LINC00707 in 786-O and 769-P cells, EdU-positive ratio was remarkably declined, suggesting the attenuated proliferative ability (Figure 2A-2C). Moreover, CCK-8 assay revealed the reduced viability in 786-O and 769-P cells transfected with si-LINC00707 relative to those transfected with si-NC (Figure 2D, 2E). As a result, silence of LINC00707 remarkably attenuated proliferative ability of ccRCC.

Knockdown of LINC00707 Suppressed the Metastatic Abilities of ccRCC

Potential influences of LINC00707 on the metastatic abilities of ccRCC were assessed by performing transwell assay. Migrative cell number was markedly reduced after transfection of si-LINC00707 in 786-O and 769-P cells (Figure 3A, 3B). Meanwhile, the invasive cell number was also reduced by silence of LINC00707 (Figure 3C, 3D). LINC00707 was proved to accelerate ccRCC to migrate and invade.

LINC00707 Activated EMT Pathway in ccRCC

EMT was first discovered in embryonic development. Later, EMT occurrence is discovered after the injury of adult epidermal cells. In the occurrence and progression of tumors, EMT leads to loss of epithelium characteristics and gain of mesenchymal cell phenotypes, thereafter, enhancing the metastatic capacities[14]. The process of EMT is regulated by a series of related transcription factors, most notably the downregulated E-cadherin, Cytokeratin, ZO-1, and upregulated N-cadherin, Vimentin, Snail1, Snail2, Twist, MMP-2, etc.[15,16]. Here, we determined expression levels of E-cadherin and N-cadherin in ccRCC.

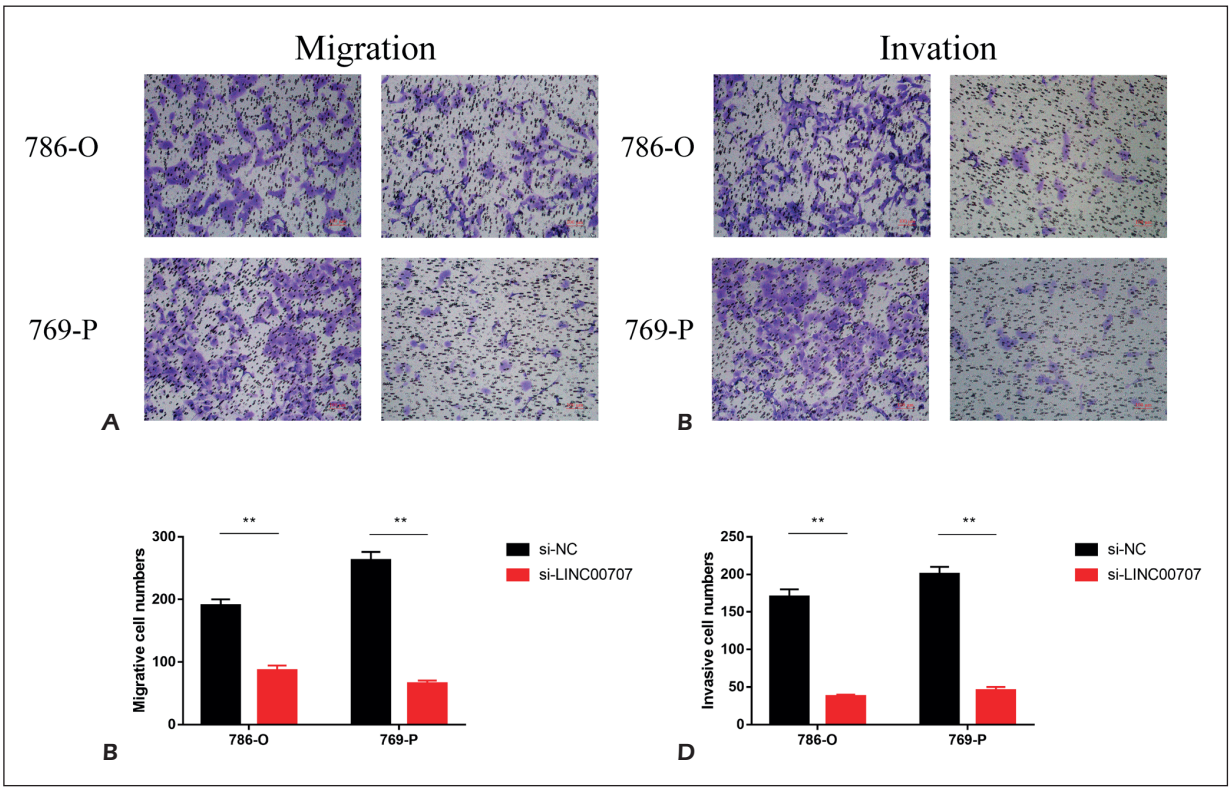

Figure 3. Knockdown of LINC00707 suppressed the migratory and invasive abilities of ccRCC. **A**, Migration in 786-O and 769-P cells transfected with si-NC or si-LINC00707 (magnification: 40×). **B**, Migrative cell number in 786-O and 769-P cells transfected with si-NC or si-LINC00707. **C**, Invasion in 786-O and 769-P cells transfected with si-NC or si-LINC00707 (magnification: 40×). **D**, Invasive cell number in 786-O and 769-P cells transfected with si-NC or si-LINC00707

Silence of LINC00707 upregulated the mRNA level of E-cadherin and downregulated N-cadherin in 786-O and 769-P cells (Figure 4A, 4B). Similar results were yielded at their protein levels (Figure 4C, 4D). It is suggested that LINC00707 was capable of activating EMT pathway, thus aggravating the progression of ccRCC.

Discussion

LncRNA exerts an important role in tumor development. It is widely involved in tumor epigenetics, signaling pathway regulation and their interactive regulation as skeleton, guiding, decoy, and signaling molecules. Abnormally expressed lncRNAs are of significance in different stages of tumor progression. They are capable of regulating cellular behaviors of tumor cells. It is reported that LINC00707 stimulates the proliferation and metastasis of gastric cancer by interacting with the mRNA stabilizing protein HuR[13]. In liver cancer, LINC00707 sponges miR-206 to upregulate CDK14, thus aggravating the disease progression[17]. By absorbing miR-370-3p to upregulate WNT2B, LINC00707 accelerates the osteogenesis of bone marrow mesenchymal stem cells[18]. In this paper, LINC00707 was upregulated in ccRCC tissues and cell lines. Silence of LINC00707 inhibited ccRCC to proliferate, migrate, and invade.

Epithelial-mesenchymal transition (EMT) is the biological process where epithelial cells are transformed into cells with mesenchymal phenotype. EMT is vital in embryonic development, chronic inflammation, tissue remodeling, cancer metastasis, and various fibrotic diseases. Its main features include downregulation of cell adhesion molecules (such as E-cadherin), cytoskeletal transformation from cytokeratin to vimentin, and the occurrence of morphological characteristics of mesenchymal cells. EMT leads to the losses of cell polarity and attachment to the basement membrane, thus enhancing the metastatic ability. It is a necessary process for tumor cells obtaining higher capacities of migration and invasiveness. To elucidate the molecular mechanism of EMT in malignant tumor cells contributes to develop therapeutic targets alleviating tumor metastasis.

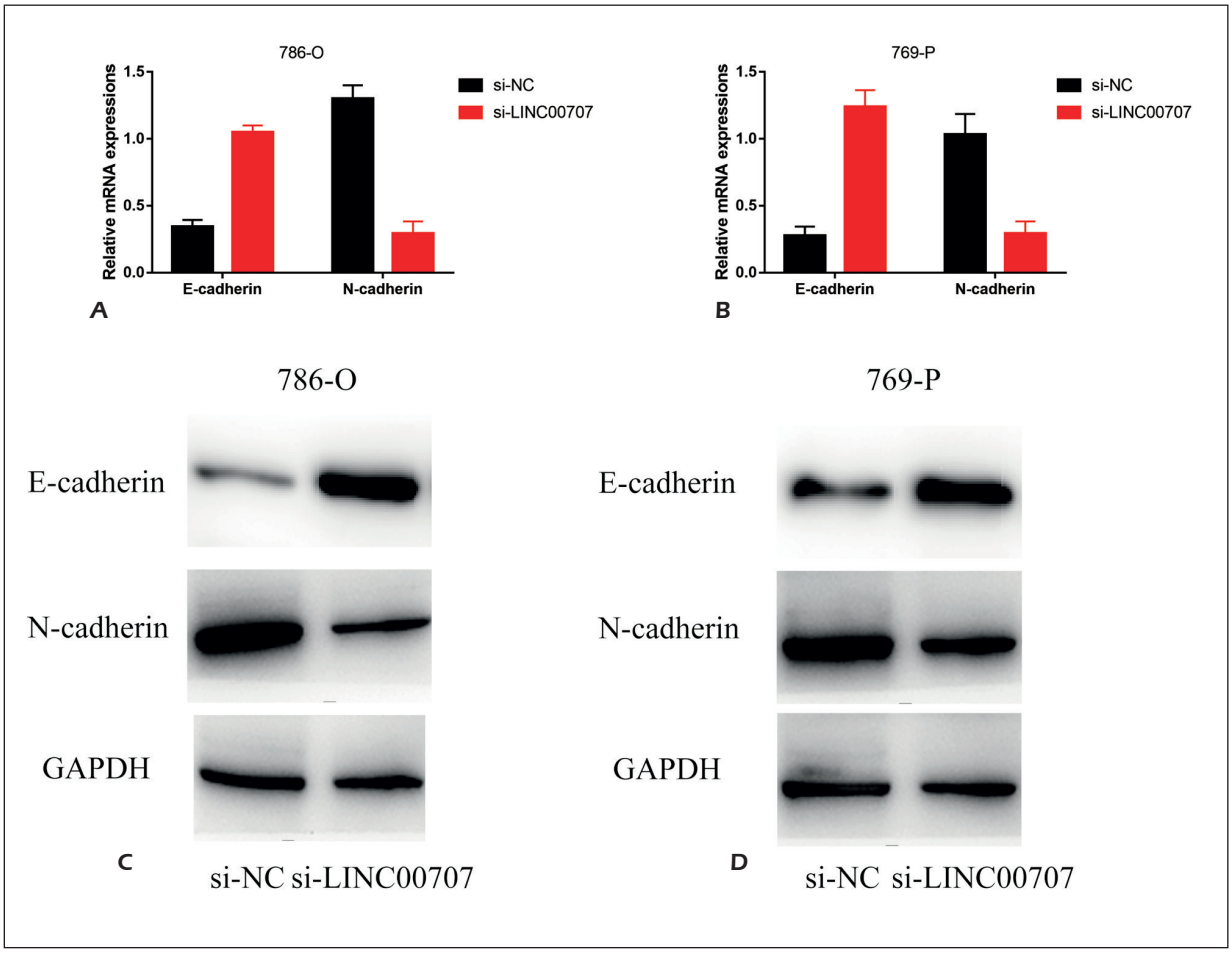

Figure 4. LINC00707 activated EMT pathway in ccRCC. **A**, mRNA levels of E-cadherin and N-cadherin in 786-O cells transfected with si-NC or si-LINC00707. **B**, mRNA levels of E-cadherin and N-cadherin in 769-P cells transfected with si-NC or si-LINC00707. **C**, Protein levels of E-cadherin and N-cadherin in 786-O cells transfected with si-NC or si-LINC00707. **D**, Protein levels of E-cadherin and N-cadherin in 769-P cells transfected with si-NC or si-LINC00707.

LncRNAs are identified to mediate EMT in tumors. For example, lncRNA OTUD6B-AS1, which is highly expressed in ccRCC, can inhibit the expressions of EMT-related proteins *via* Wnt/β-catenin[19]. LncRNA ATB promotes EMT of breast cancer by sponging miR-200c[20]. Up-regulated LINC01296 in pancreatic ductal adenocarcinoma indicates a poor prognosis and promotes cell metastasis by affecting EMT[21]. We showed that knockdown of LINC00707 upregulated E-cadherin and downregulated N-cadherin in ccRCC cells at both mRNA and protein levels. It is indicated that LINC00707 accelerated the progression of ccRCC by activating EMT pathway. However, we failed to clarify the molecular mechanism of LINC00707 in influencing the EMT of ccRCC, which requires further explorations.

Conclusions

This study showed that LINC00707 is upregulated in ccRCC, which could promote cancer cells to proliferate, migrate, and invade. LINC00707 accelerates the progression of ccRCC by activating EMT pathway. LINC00707 may be a novel hallmark for predicting the progression of ccRCC.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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