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Long non-coding RNA ENST00000434223 inhibits the progression of renal cancer through Wnt/hygro-catenin signaling pathway

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Abstract. – OBJECTIVE: The purpose of this study was to determine the function of long non-coding RNA (LncRNA) ENST00000434223 (Lnc ENST) in renal carcinoma, and to explore the potential molecular mechanism.

PATIENTS AND METHODS: Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expressions of lncRNA ENST00000434223 and Wnt/β-catenin pathway-related mRNAs in tissues and cells of renal cancer. Chi-square test was performed to figure out the relationship between lncRNA ENST00000434223 and clinic-pathologic features of renal cancer patients. Besides, si-NC, si-ENST00000434223, pcDNA-NC and pcDNA-ENST00000434223 were transfected into renal cancer cells. The proliferative ability, metastasis and invasiveness of cells were detected using Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Lastly, the activation of the Wnt/hygro-catenin signal transduction pathway was evaluated by TOP/FOP Wnt Luciferase reporter assay and Western blot.

RESULTS: The expressions of Wnt2b and β-catenin were significantly increased in renal carcinoma, while E-cadherin was markedly down-regulated. Lowly expressed ENST00000434223 was involved in the poor prognosis of patients with renal cancer. In addition, down-regulating ENST00000434223 could enhance the viability, metastasis and invasiveness of renal cancer cells. However, overexpressing ENST00000434223 remarkably weakened the above cell functions. At the same time, interference or overexpression of ENST00000434223 could affect the expression level of proteins related to the Wnt/β-catenin signal pathway.

CONCLUSIONS: LncRNA ENST00000434223 inhibits the progression of renal cancer through the Wnt/shell-catenin signal pathway.

Key Words:

LncRNA ENST, Wnt/β-catenin signal pathway, Renal carcinoma, Molecular mechanism.

Introduction

Renal cancer is the most common urological tumor, ranking 16th in tumor-induced deaths[1]. Conventional radiotherapy, chemotherapy and hormone therapy exhibit a poor effect on renal cancer, resulting in limited treatment[2]. At present, radical nephrectomy is still the most effective method for the treatment of renal cell carcinoma[3,4]. However, still 40% of the patients experience recurrence after surgery. Meanwhile, these combined factors lead to the poor prognosis of patients with renal carcinoma. Therefore, it is particularly important to investigate the main mechanism of the occurrence and development of renal cancer and to improve its treatment[5].

Studies have shown that long non-coding RNA (lncRNA) exerts a vital effect on many life activities, such as dose compensation effect, epigenetic regulation, cell cycle regulation and cell differentiation regulation. Meanwhile, it has become a hotspot in biomedical research[6,7]. At present, many lncRNAs have been found to be involved in the occurrence and evolution of cancer. They can also affect cancer invasion and metastasis[8]. For example, lncRNA H19[9,10] in colorectal cancer and nasopharyngeal cancer, SPRY-IT1[11] in melanoma, HOTAIR, AFAP1-AS1 and NKILA[12-14] in lung cancer, lncRNA-RoP[15] in breast cancer, and lncRNA ATB[16] in liver cancer can all accelerate the invasiveness of tumor cells. In recent years, research has already highlighted the importance of lncRNAs in cancer biology. Although more studies are focused on lncRNAs, only a small proportion of the function of lncRNAs has been elucidated. A recent work has found that long-chain RNA ENST00000434223 is expressed in early lung adenocarcinoma. Moreover, overexpressing ENST00000434223 can significantly in-

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hibit the proliferative capacity of non-small cell carcinoma[17]. However, its research in the renal tumor has not been reported yet. Therefore, it is of great significance to explore the mechanism of lnc ENST00000434223 in renal cell carcinoma.

The Wnt/β-catenin signal pathway is a classic pathway widely associated with the regulation of multiple biological activities of tumor cells. Relevant studies[18] have found that activation of the Wnt/β-catenin signal pathway is involved in the occurrence, metastasis, drug resistance and other malignant behaviors of tumor cells. In skin cancer[19], silencing of this pathway can lead to apoptosis of CSCs and reduction of tumors. In colorectal cancer cells, the upregulation of transcriptional activation complex of β-catenin/Tcf or nuclear accumulation of this protein has been found to cause the activation of target genes. Meanwhile, this can inhibit normal cell differentiation, leading to the occurrence and progression of tumors[20]. Studies have also demonstrated that the Wnt/β-catenin pathway exerts a significant influence on renal cancer. Shiina et al[21] have indicated that the activation of the Wnt/β-catenin pathway promotes the formation and development of renal cancer. In renal clear cell carcinoma and papillary carcinoma, some researchers have proved that β-catenin transposition and intracellular activation of oncogene c-myc can lead to the occurrence and progression of renal carcinoma[22,23]. However, the specific mechanism of the correlation between lncRNA ENST00000434223 and the Wnt/β-catenin signaling pathway in renal cancer remains unclear.

Therefore, this work first explored the role of lncRNA ENST00000434223 in renal cancer. We also studied the effects of ENST00000434223 on the proliferation and invasiveness of renal cancer cells, as well as the role related to the Wnt/β-catenin signal pathway. Our study might provide new ideas and effective targets for the treatment of renal cancer.

Patients and Methods

Tissue Samples and Clinical Data

Cancer tissues and para-cancerous tissues were collected from 60 renal cancer patients. All fresh tissue samples were rapidly frozen in liquid nitrogen and stored at -80°C for subsequent experiments. At the same time, clinical data of the patients were collected, including gender, age, tumor size, tumor node metastasis (TNM) stage, lymph node metastasis and tissue differentiation. This study was approved by the Ethics Committee of the Gansu Provincial Hospital. Signed informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Human ccRCC-derived cell lines (786-O and ACHN) and normal human proximal tubular epithelial cell line (HK-2) were selected for cell models. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% standard fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in a 37°C, 5% CO_2 incubator. The culture medium was changed once a day. When the density was up to 80%, cell passage was carried out or a corresponding subsequent experiment was performed. Si-NC or si-ENST00000434223 (sequence: GGAGGGUGCUUGACAAUAAUU) was transfected into 786-O cell line. To overexpress ENST00000434223, pcDNA vector was transfected into ACHN cells according to the instructions of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from kidney cancer cell lines in strict accordance with pre-chilled TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction was carried out according to PrimeScript RT reagent Kit with gDNA Eraser TaKaRa Code: DRR047A Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. Complementary deoxyribose nucleic acid (cDNA) obtained by reverse transcription was diluted to an appropriate multiple. Subsequently, cDNA was subjected to experiments according to the Real Time-quantitative premix instructions. Briefly, an appropriate amount of cDNA, primers, premix and ultra-pure water were mixed into 20 systems in the 8-tube. After mixing and centrifugation, Polymerase Chain Reaction (PCR) amplification was conducted according to the recommended parameters in the manual. Fluorescence signal was collected and analyzed. Primer sequences used in this study were as follows: ENST000000434223 (Forward) 5'-GCAAGTGTTGAAGGACGACGAT-3', ENST000000434223 (Reverse) 5'-CAAGGAGGCATACACGGAGTT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward) 5'-AGAAGGCTGGGGCTCATTTG-3', GAPDH (Reverse) 5'-AGGGGCCATCCACAGTCTTC-3'.

Western Blot

RCC cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor. The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% non-fat milk, the membrane was incubated with primary antibodies of anti-PTEN (dilution 1:1000) and anti-GAPDH (dilution 1:2000). The next day, the membrane was incubated with Horse Reddish Peroxidase (HRP) secondary antibodies conjugated by horseradish peroxidase for 1 h. Electrochemiluminescence (ECL) detection system (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the signal. Quantity One software was used to quantify the signal by optical density measurement.

Dual-Luciferase Reporter Gene Assay

786-O cells were first seeded into 24-well plates and co-transfected with TOP/FOP Flash plasmid and Renilla TK-Luciferase vector. 48 hours after transfection, cells were harvested and lysed for Luciferase assay (Promega, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were first seeded into 96-well plates at a density of 2000 cells per well and incubated at 37°C. 6 h, 24 h, 48 h and 72 h after inoculation, 10 μL of Cell Counting Kit-8 reagent (CCK-8; Dojindo, Kumamoto, Japan) was added to each well, followed by incubation for 1.5 h. 6 replicates were set in each group, including the blank control group. The absorbance of each well at the wavelength of 450 nm was measured by a microplate reader. Finally, the cell growth curve was plotted.

Transwell Assay

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was preliminarily melted at 4°C. The mixture was diluted 1:3 with serum-free medium 200. Then, the cells were seeded into the upper chamber at a density of 1×10^5 with serum-free medium.

Statistical Analysis

GraphPad Prism 6 (La Jolla, CA, USA) was used for all statistical analysis. *t*-test was performed to compare the difference of measurement data between the two groups. The Chi-square test was used for the comparison of classified data. $p<0.05$ was considered statistically significant: $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

LncRNA ENST00000434223 is Lowly Expressed in Renal Cancer Tissues and Cells, Which is Associated with Poor Prognosis

The expression of ENST00000434223 in renal cancer tissues and para-cancerous tissues, as well as renal cancer cell lines was detected by qRT-PCR. The results indicated that ENST00000434223 was lowly expressed in renal carcinoma tissue (Figure 1A) when compared with paired para-cancerous tissues. In addition, the expression level of ENST00000434223 786-o and ACHN cells was notably lower than that of proximal tubule epithelial cell line hk-2 (Figure 1B). We further analyzed the relationship between the expression of ENST00000434223 and clinic-pathological features in 60 patients with renal cancer. According to the median expression of ENST00000434223 in these patients, they were divided into the high expression group and low expression group. As shown in Table I, it was found that the expression of ENST00000434223 was closely related to tumor size, tumor stage and lymph node metastasis ($p<0.05$). Furthermore, the relationship between the expression of ENST00000434223 and the prognosis of renal cancer patients was studied by the Kaplan-Meier survival analysis. The results indicated that the overall survival rate of patients in ENST00000434223 low expression group was significantly lower than that of the high expression group (Figure 1C). These data showed that the expression of ENST00000434223 was down-regulated in renal carcinoma.

LncRNA ENST00000434223 Inhibits Proliferation of Renal Carcinoma Cells

To elucidate the role of ENST00000434223 in the progression of renal carcinoma, si-ENST00000434223 and pcDNA-ENST00000434223 were transfected into cells to reduce and increase the expression of ENST00000434223 (Figure 2A and 2B), respectively. CCK8 assay was used to detect cell viability. It was found that interference with ENST00000434223 expression markedly promoted the proliferation of renal cancer

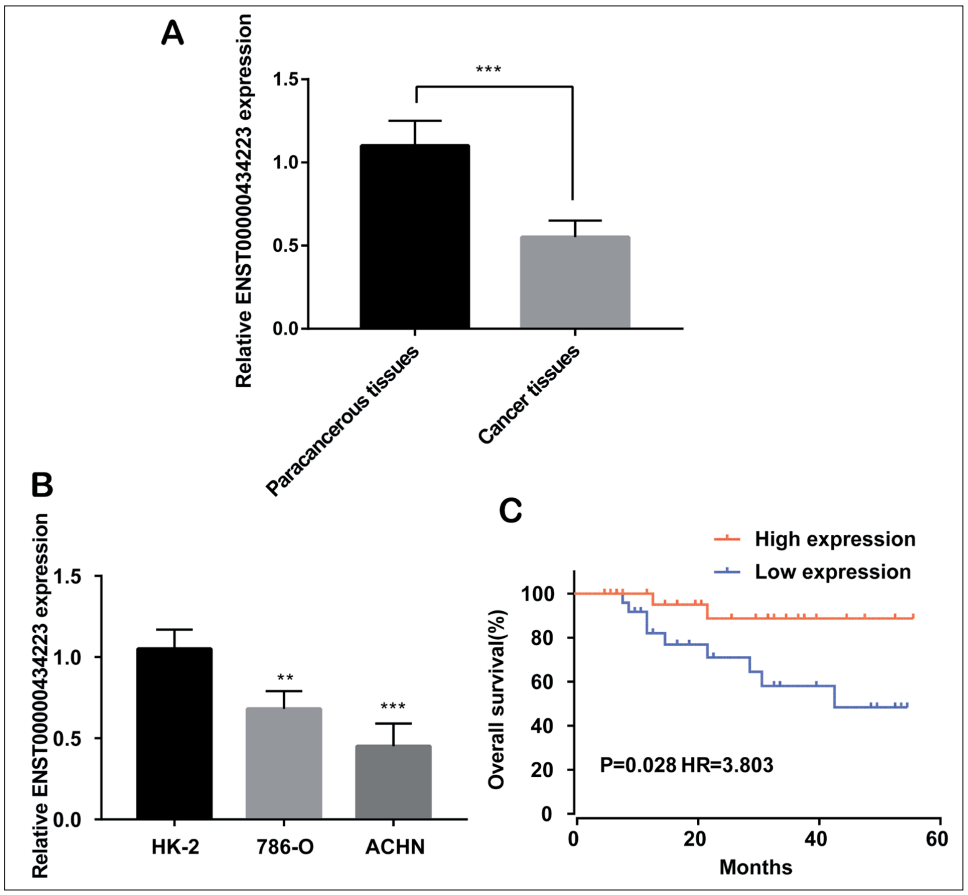

Figure 1. LncRNA ENST00000434223 was lowly expressed in renal carcinoma. ***A,*** LncRNA ENST00000434223 expression in renal cell carcinoma tissues was significantly lower than that of the adjacent tissues. ***B,*** The expression levels of lncRNA ENST00000434223 in two ccRCC cell lines (786-O and ACHN) and one normal near-end tubular epithelial cell line (hk-2) were detected by qRT-PCR. ***C,*** The overall survival rate of patients with high expression of lncRNA ENST00000434223 was markedly higher than that of patients with low expression of lncRNA ENST00000434223.

Table I. Correlation between lncRNA ENST00000434223 expression and clinicopathological features in renal cancer patients (n=60).

Clinicopathological features	No. of cases	ENST00000434223 expression		*p*-value
		Low (n = 30)	High (n = 30)	
Age (years)				0.301
< 60	32	14	18	
≥ 60	28	16	12	
Gender				0.796
Male	29	15	14	
Female	31	15	16	
Diameter (cm)				0.018*
< 5 cm	25	8	17	
≥ 5 cm	35	22	13	
TNM stage				0.012*
I-II	19	5	14	
III-IV	41	25	16	
Lymph node metastasis				0.020*
No	27	9	18	
Yes	33	21	12	
Degree of differentiation				
Well and moderately	30	14	16	0.606
Poorly	30	16	14	

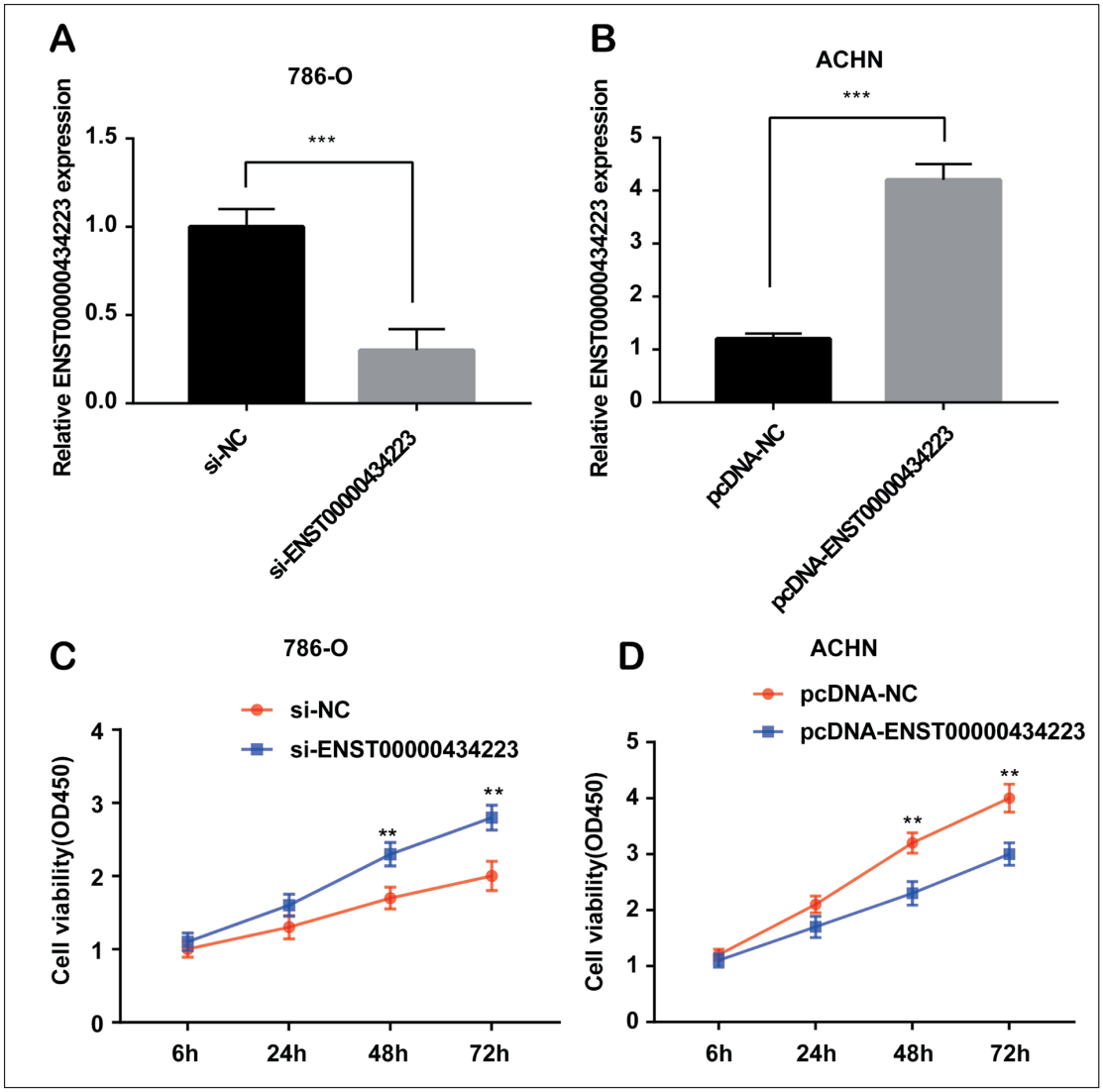

Figure 2. LncRNA ENST00000434223 inhibits proliferation of renal cancer cells. ***A,*** The expression of lncRNA ENST00000434223 was detected by qRT-PCR in 786-o cells. ***B,*** After overexpression of lncRNA ENST00000434223 in ACHN cells, lncRNA ENST00000434223 expression was up-regulated. ***C,*** The results of CCK8 experiment showed that the activity of 786-O renal cells in si-ENST00000434223 group was remarkably increased when compared with si-NC group. ***D,*** CCK8 experiments showed that the overexpression of lncRNA ENST00000434223 significantly decreased the viability of ACHN cells.

cells (Figure 2C). However, overexpressing ENST00000434223 obtained the opposite results (Figure 2D).

LncRNA ENST00000434223 Inhibits Metastasis and Invasiveness of Renal Cancer Cells

We further examined the influence of ENST00000434223 on the invasiveness of renal cancer cells *via* transwell assay. The results demonstrated that the down-regulation of ENST00000434223 in 786-O cells significantly enhanced cell invasive abilities when compared with the si-NC group (Figure 3A). In addition, after overexpression of ENST00000434223 in ACHN cells, cell migration and invasiveness were remarkably weakened compared with the normal control group (Figure 3B). The above results suggested that ENST00000434223 inhibited migratory and invasive capacities of 786-O and ACHN renal cancer cells.

ENST00000434223 Inhibits the Wnt/β-Catenin Pathway

To clarify the relationship between ENST00000434223 and the Wnt/β-catenin sig-

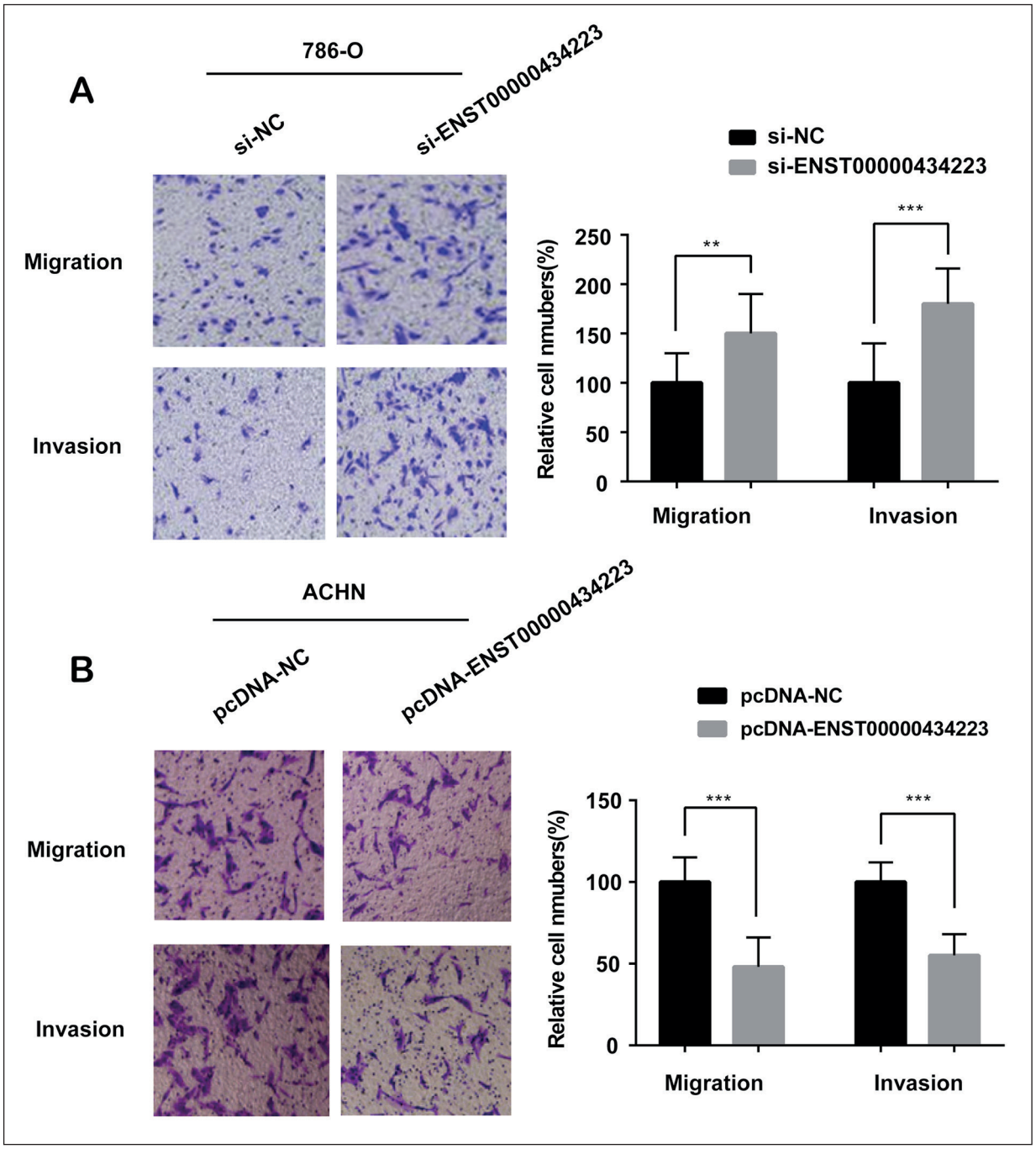

Figure 3. LncRNA ENST00000434223 inhibits migration and invasion of renal cancer cells. ***A,*** The transwell assay showed that after transfection of si-ENST00000434223 in 786-O renal cells, the migration and invasion abilities were markedly enhanced (magnification: 40×). ***B,*** PcDNA -ENST00000434223 was transfected into ACHN cells (magnification: 40×).

nal pathway, TOP/FOP flash reporter gene assay was performed to evaluate the effect of ENST00000434223 in 786-O cells. The results showed that the Wnt/β-catenin signal pathway was activated when ENST00000434223 was silenced. Furthermore, the expression of the Wnt/β-catenin-associated mRNAs in renal tumor and corresponding normal tissues was detected by qPCR. It was found that, compared with paired normal tissues, the level of N-cadherin was significantly increased, whereas E-cadherin was decreased (Figure 4B). Western blot was further used to detect the protein expression levels of molecules related to the Wnt/β-catenin signal pathway after interfer-

ence and overexpression of ENST00000434223, respectively. The results showed that after knockdown of ENST00000434223, the expressions of Wnt2b, β-catenin, cyclinD1, n-cadherin, Vimentin and snail were markedly increased, whereas E-cadherin was markedly decreased. In addition, the opposite results were observed after ENST00000434223 overexpression

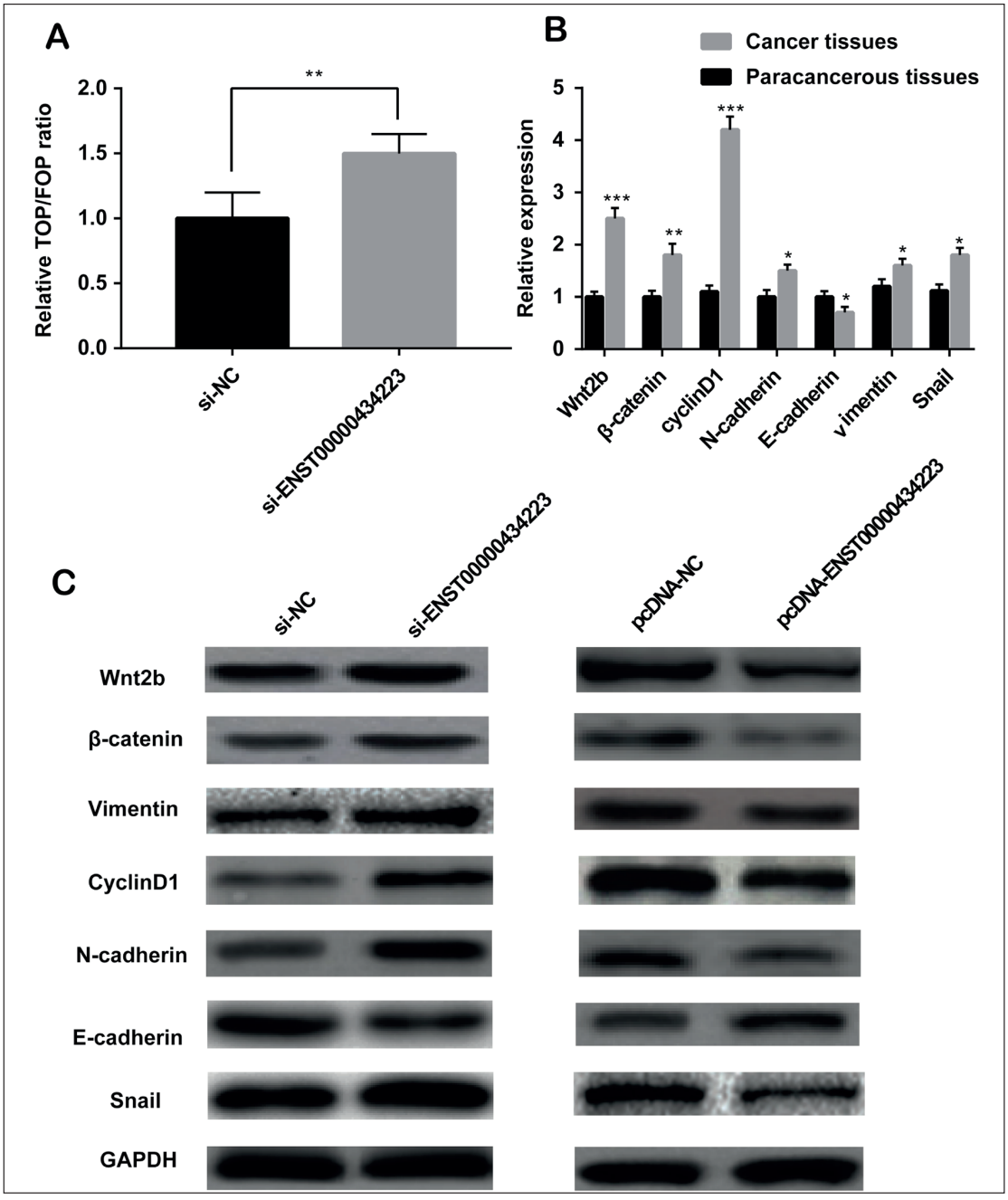

Figure 4. ENST00000434223 inhibits the activation of the Wnt/β-catenin signal pathway. ***A,*** Luciferase reporter gene assay was performed using TOPflash vector to detect the activity of the β-catenin transcription factor/lymphatic enhancer binding factor (TCF/LEF) promoter in 786-O cells. ***B,*** Western blot assay was used to detect the protein expression of the Wnt/β-catenin signal pathway related molecules in para-cancerous and renal cancer tissues. ***C,*** Western blot showed that after transfection of si-ENST00000434223, the protein expressions of Wnt2b, β-catenin, cyclinD1, N-cadherin, Vimentin and snail were significantly increased, while E-cadherin was remarkably decreased. After transfection of pcDNA-ENST00000434223, the protein expressions of Wnt2b, β-catenin, cyclinD1, N-cadherin, Vimentin and snail were decreased, while E-cadherin was increased.

(Figure 4C). The above results indicated that ENST00000434223 might have an influence on the activity of the Wnt/β-catenin signal pathway.

Discussion

There are about 5-10 kidney cancer patients per million people, accounting for 2-3% of all adult malignant tumors[24,25]. Therefore, it is urgent to explore the molecular mechanism of the occurrence and development of renal cancer. Meanwhile, we also need to find efficient biomarkers and specific therapeutic targets for improving the survival rate of these patients.

In recent years, as a new molecule with spatiotemporal specificity and good stability, lncRNA has gradually become the focus of contemporary biomedical research. Evidence has indicated that lncRNAs are especially indispensable in the pathogenesis of renal cell carcinoma[26-28]. GAS5, HOTAIR, MEG3, MALAT1 and SRCC, for example, have been proved to be involved in renal carcinoma occurrence[29-33]. As a lncRNA, ENST00000434223 is related to the proliferative capacity, invasiveness and EMT of tumor cells. Currently, it has only been found in non-small cell lung cancer and gastric cancer that the overexpression of ENST00000434223 inhibits the viability of cancer cells, eventually leading to EMT reversal[34,35]. However, the exact role and mechanism of lncRNA ENST00000434223 in renal tumor remains elusive. In this work, we found that the expression of ENST00000434223 in renal cancer tissues was significantly lower than that of corresponding para-cancerous tissues. Besides, compared with normal renal carcinoma cell lines, ENST00000434223 level in renal carcinoma cell lines of 786-o and ACHN was markedly down-regulated. The results of a series of cellular experiments demonstrated that the overexpression of ENST00000434223 remarkably inhibited the proliferation, migration and invasion of renal carcinoma cells. These results revealed that ENST00000434223 played an important inhibitory role in the progression of renal carcinoma.

The Wnt signal pathway is an important conservative pathway commonly found in mammals. It plays a very important regulatory role in cell growth, development, apoptosis, differentiation, and cell homeostasis[36]. E-cadherin can also be used as a negative modulator of the Wnt/β-catenin pathway to affect the localization of β-catenin in cells. E-cadherin binds to β-catenin, which remains in the cytoplasm. Promoter methylation leads to the silencing of E-cadherin coding gene CDH1. This occurs in various cancers, which also mediates abnormal activation of the Wnt/β-catenin signaling pathways[37]. In addition, studies have found that the Wnt signal pathway is also related to a variety of diseases, such as metabolic diseases[38], tumors[39,40] and others. Previous studies have shown that the Wnt/β-catenin pathway is closely related to the occurrence and development of renal cancer. Huang et al[41] have found that CCAT2 overexpression affects the biological behavior of ccRCC cells by activating the Wnt/β-catenin signal transduction. Chen et al[42] have indicated that miR-195-5p overexpression inhibits the Wnt/β-catenin pathway, thereby suppressing the proliferation of renal cell carcinoma. However, few studies have focused on the relationship between lncRNA ENST00000434223 and the Wnt/β-catenin signal pathway in renal cancer. In our work, it was found that interfering lncRNA ENST00000434223 could activate the Wnt/β-catenin signal pathway, resulting in an elevated level of N-cadherin and decreased E-cadherin. Furthermore, this could promote the progression of renal cancer.

Conclusions

We detected that lncRNA ENST00000434223 expression was down-regulated in renal cancer tissues and cells. Low expression of ENST00000434223 was associated with poor prognosis of these patients. In addition, highly expressed ENST00000434223 could significantly inhibit the proliferation, invasion and migration of cancer cells by inactivating the Wnt/β-catenin signal pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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