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LSINCT5 activates Wnt/β-catenin signaling by interacting with NCYM to promote bladder cancer progression

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ABSTRACT

Accumulating evidence has indicated that long non-coding RNAs (lncRNAs) are critically involved in tumor progression. In current study, we reported a novel lncRNA signature correlated with bladder cancer development. Particularly, the lncRNA long stress-induced noncoding transcript 5 (LSINCT5) is significantly upregulated in human bladder cancer cell lines and tumor specimens. Meanwhile, high LSINCT5 expression correlates with poor prognosis, enhances tumor sphere formation and invasion *in vitro*. *In vivo* xenograft tumor growth is also elevated by LSINCT5 overexpression. Mechanistic investigations showed that LSINCT5 could physically interact with NCYM, a *de novo* gene product from the MYCN cis-antisense RNA and inhibit GSK3β activity leading to enhanced Wnt/β-catenin signaling activation and epithelial mesenchymal transition (EMT). Taken together, our findings have created a novel LSINCT5 mediated process which facilitates bladder cancer progression and may provide a potential target for therapeutic intervention.

1. Introduction

The bladder cancer (BC) is highly recurrent and among the leading cause of death, accounting for ~3% of total cancer incidence [1]. Urothelial carcinoma belongs to the most common type of bladder cancer with transitional carcinoma [2]. The routine treatment for bladder cancer patients is endoscopic resection while the systemic chemotherapy at present is only efficient in a fraction of patients and bladder cancer patients suffer from a relatively low five-year survival [3]. Therefore, identifying novel molecular pathways or mechanisms responsible for bladder cancer development plays critical role in anticancer therapy.

The long noncoding RNA (lncRNA) is a kind of noncoding RNAs with >200 nucleotides in length [4]. Although there is no protein-coding capacity for lncRNA, it has been implicated in diverse processes during cancer progression such as initiation and metastasis [5]. Accumulating evidences have shown that lncRNAs may serve as tumor suppressors or oncogenes during bladder cancer incidence. For example, lncRNA-GAS5 inhibits chemokine (C-C) ligand 1 (CCL1) expression to inhibit bladder cancer proliferation [6]. Instead, the terminal differentiation-induced noncoding RNA (TINCR) could promote bladder cancer progression and Theophylline controllable TINCR expression may reduce the malignant phenotypes [7]. The long stress-induced noncoding transcript (LSINCT) 5 is an intergenic lncRNA between *IRX4* and *IRX2* genes and is substantially upregulated in breast and ovarian cancer cells [8]. LSINCT5 also predicts negative prognosis in gastric cancer [9] and mediates myocardial cell apoptosis [10]. However, the potential role of LSINCT5 and underlying mechanism in bladder cancer progression remains elusive.

In this work, by lncRNA profiling we identify that LSINCT5 overexpression is a characteristic change in bladder cancer and indicates poor survival. LSINCT5 plays an oncogenic role both *in vitro* and *in vivo*. Furthermore, by RNA immunoprecipitation (RIP) followed by mass spectrometry (MS) we found that LSINCT5 could interact with NCYM, a *de novo* evolved gene product from a MYCN cis-antisense RNA. NCYM can inhibit GSK3β activity leading to activated Wnt/β-catenin signaling and accelerated epithelial mesenchymal transition (EMT). Collectively, our results have presented the first evidence about the oncogenic role of LSINCT5 in bladder cancer through regulating Wnt/β-catenin pathway.

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2. Materials and methods

2.1. Cell lines and reagents

All bladder cancer cell lines (UBC-40, T24, SW780, RT4 and J82) and the normal human uroepithelial SV-HUC-1 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology. Cell lines were maintained in humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM, no. D5796, Sigma) plus 5% fetal bovine serum (FBS, no. F8067, Sigma) and 100 μg/ml streptomycin (no.V900929, Sigma). Antibodies for Vimentin (no.SAB4301438), Ecadherin (no.5085), GAPDH (no.SAB1403850), Lamin B1 (no. SAB1306342), GSK3β (no. SAB5300175) were obtained from Sigma. NCYM antibody (no.sc-168720) was purchased from Santa Cruz. Antibodies for p-β-catenin (Ser33/37/Thr41, no.9561), p-β-catenin (Thr41/Ser45, no.9565) and β-catenin (no.8480) were purchased from Cell Signaling Technology.

2.2. In situ hybridization and subcellular fractionation

Bladder cancer cells were fixed in 4% formaldehyde for 15 min and washed with PBS. Fixed cells were then treated with pepsin (1% in 10 mM HCl) and dehydrated through ethanol. The samples were further incubated with 40 nM FISH probe in hybridization buffer (100 mg/mL dextran sulfate, 10% formamide in 2 × SSC) at 80 °C for 1 min. Hybridization was performed at 55 °C for 2 h and the slide was washed and dehydrated. Finally, the air-dried slide was covered with Prolong Gold Antifade Reagent with DAPI for detection. Sections were also cut and stained with hematoxylin-eosin (HE). RNA FISH probes were designed and synthesized by Bogu Co., Ltd (Shanghai, China). Nuclear and cytosolic fractions were separated using the PARIS Kit (Life Technologies, Shanghai, China).

2.3. Human samples

The human bladder cancer specimens (N = 108) and matched normal adjacent tissues were collected from Liaocheng People's Hospital between May 2012 and February 2013. The specimens were immediately frozen in liquid nitrogen and stored at −80 °C refrigerator before use. None patients have received preoperative chemotherapy or radiotherapy. The experimental protocols were approved by the Ethical Review Committee of the Liaocheng People's Hospital. Informed consent was obtained from all patients.

2.4. RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNAs were extracted using the TRIzol reagents (Invitrogen) according to the manufacturer's protocols. Quantifying RNA was performed using Nanodrop 2000 (Thermo Scientific). The cDNA was synthesized by PrimeScript RT Reagent Kit (TaKaRa) and qRT-PCR was done using SYBR Green Premix Ex Taq (TaKaRa). Reactions were performed with ABI PRISM® 7000 Sequence Detection System (Applied Biosystem) according to the manufacturer's instructions.

2.5. RNA pulldown assay

Briefly, Biotin-labeled RNAs were transcribed with Biotin RNA labeling Mix (no. 2147483647, Roche) and T7 RNA polymerase and then purified with the RNeasy Mini Kit (no. 74104, Qiagen). Total RNAs were annealed to a proper secondary structure, mixed with cytoplasm extract by RIP buffer, and then incubated at 20 °C for 30 min. Streptavidin magnetic beads were incubated at 20 °C for 60 min. Trizol reagent was used to extract the beads for quantitative PCR (qPCR). Electrophoresis was used to separate specific bands for subsequent mass spectrometry (MS). Mass spectrometry was performed in National Laboratory of Protein Engineering and Plant Genetic Engineering of Peking University.

2.6. Western blot

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and protease cocktail inhibitor I (Calbiochem, San Diego, CA). Then, lysates were separated with SDS-PAGE (8% polyacrylamide) and transferred to polyvinylidene difluoride membranes (PVDF, no. P2438, Sigma). Blots were blocked TBST containing 4% skimmed milk and then incubated with antibodies overnight at 4 °C. The membranes were again washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (no. 1662408, BioRed) for 1 h at 20 °C. After three washes by TBST, immunoreactive bands were detected using enhanced chemiluminescence (Santa Cruz Biotechnology) followed by exposure on BioMax film (Kodak).

2.7. RNA immunoprecipitation (RIP)

RIP was performed using the Imprint® RNA Immunoprecipitation Kit (Sigma) according to the manufacturer's protocols. Total RNAs and precipitation with the isotype control (IgG) were simultaneously evaluated. 15 μg NCYM antibodies were used at the supernatant. Precipitated RNAs were separated using the protein G beads and then quantified with qPCR.

2.8. Plasmid construction and transfection

The full-length cDNA of LSINCT5 was synthesized by Invitrogen (Shanghai, China) and inserted into the lentiviral expression vector pWPXL. NCYM was inserted into pCMV-Flag vector for overexpression (abbreviated as "oe"). Cells were co-transfected with pWPXL-LSINCT5 and the lentiviral vector packaging system using Lipofectamine 2000. Small hairpin RNAs (shRNAs) for LSINCT5 and NCYM were designed and purchased from Invitrogen (Shanghai, China). The final constructs were verified by sequencing. The shRNA sequences were shown in Table S1.

2.9. The lncRNA microarray, transwell invasion assay, tumor sphere formation, immunoprecipitation and in vivo tumorigenesis model

Please refer to supplemental materials.

2.10. Statistical analysis

All data were shown as mean ± SD. The student's *t*-test was used to identify the statistical significance between two groups. One-way ANOVA was used for comparison among multiple groups followed by LSD *post hoc* test. 'High' or 'low' expression was determined by whether the expression was higher or lower than the median value. The log-rank test was used to evaluate the Kaplan-Meier survival plot. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. LSINCT5 is specifically upregulated in bladder cancer

To identify potential oncogenic lncRNAs in bladder cancer tumorigenesis, we performed lncRNA profiling between SV-HUC-1/T24 pairs [11] and from paired tumor/normal tissues. Two

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