Novel IncRNA LINC01614 Facilitates Bladder Cancer Proliferation, Migration and Invasion Through the miR-217/RUNX2/Wnt/β-Catenin Axis

Background: LncRNA plays a vital role in tumorigenesis and development. This study aimed to explore the novel lncRNA affecting bladder cancer progression.

Methods: The open-access data of bladder cancer patients, including transcriptome profiles and corresponding clinical information were all obtained from The Cancer Genome Atlas database. All the statistical analysis were performed using R software, SPSS and GraphPad Prism 8. CCK8, colony formation, apoptosis detection and tumorigenicity assay were used to assess cell proliferation ability. Transwell assay and wound-healing assay were used to evaluate cell proliferation ability. Transwell assay and wound-healing assay were used to evaluate cell proliferation ability.

Results: Our result showed that the lncRNA LINC01614 was highly expressed in bladder cancer tissue and cell lines. Meanwhile, patients with high LINC01614 expression level tend to have poor clinical features and shorter survival time. Further experiments demonstrated that the inhibition of LINC01614 could significantly hamper the proliferation and invasion of bladder cancer cells. Then, we found that the LINC01614 could regulate RUNX2 expression through miR-137. GSEA analysis indicated that the Wnt/β-catenin signaling pathway might be the downstream pathway of LINC01614. Further experiments showed that the LINC01614 act as an oncogene in bladder cancer partly depending on the RUNX2/Wnt/β-catenin axis, making it an underlying therapeutic target.

Conclusion: In all, LINC01614 facilitates bladder cancer cells proliferation, migration and invasion through the miR-217/RUNX2/Wnt/β-catenin axis.

Keywords: LINC01614, bladder cancer, lncRNA, Wnt/β-catenin

Introduction

Bladder cancer is the ninth prevalent malignant tumor and responsible for about 570,000 newly diagnosed bladder cancer cases and 210,000 deaths worldwide in 2020.1,2 Overall, nonmuscle-invasive bladder urothelial carcinoma accounts for approximately three-quarters of bladder cancer and the others are muscle-invasive bladder cancer.3 Despite many rapid advances that have been made in the diagnostic modalities and standard treatments of bladder cancer patients, the recurrence and death rate remains unsatisfactory and the 5-year overall survival rate is only 50–60%.4 Therefore, it is essential to explore more effective targets as tumor biomarkers to predict prognosis and guide therapy.

Long noncoding RNA (lncRNA), located in the nucleus or cytoplasm, is a noncoding RNA with a length of more than 200 nucleotides.5 Accumulating evidence has revealed that lncRNA expression changes with the specific life
process, which serves as the regulator of mRNA and protein. More and more attention has been focused on the link between IncRNA and tumor initiation, maintenance and metastasis. For example, Wu et al demonstrated that the IncRNA IncHDAC2 was highly expressed in hepatocellular carcinoma and played a critical role in promoting tumor progression through activating Hedgehog signaling. A recent study revealed that the IncRNA MALAT1 could interact and inactivate the transcription factor TEAD to prevent the link between TEAD and its co-activator YAP, thereby inhibiting breast cancer metastasis. Moreover, Dong et al indicated that the IncRNA TINC activated by H3K27 acetylation could promote Trastuzumab resistance and epithelial-mesenchymal transition in breast cancer. In breast cancer, Vishnubalaji et al demonstrated that the LINC01614 was an adverse prognosis marker, which was regulated by TGF-β and FAK signaling. Wang et al found that the LINC01614 was up-regulated in glioma and could promote cancer progression through miR-383/ADAM12 axis. Another aspect, Liu et al revealed that LINC01614 might inhibit lung cancer through miR-217/FOXP1 axis. Thus, the potential role of IncRNA might bring novel insights for the diagnosis and treatment of bladder cancer.

The rapid development of next-generation has produced large volumes of genomic data, which provides excellent convenience for researchers. Our study first identified LINC01614 as the interesting gene due to its clinical correlation and high expression level in bladder cancer based on the data obtained from The Cancer Genome Atlas (TCGA) database. Further experiments showed that the knockdown of LINC01614 could significantly inhibit the progression of bladder cancer. Meanwhile, we found this cancer-promoting effect might partly rely on the downstream RUNX2/Wnt-β-catenin axis. In all, our data demonstrated that LINC01614 might be a promising therapeutic target for bladder cancer.

Methods and Materials

Bioinformatic Analysis

The public gene-expression profile data and clinical information of bladder cancer were downloaded from the TCGA-GDC (https://portal.gdc.cancer.gov/). All the transcript data were annotated using the reference file “Homo_sapiens.GRCh38.gtf” obtained from the Ensembl website (http://asia.ensembl.org/index.html). Before analysis, the data were preprocessed with the following steps: 1) missing value completion; 2) data normalization; 3) excluding unqualified samples. Gene Set Enrichment Analysis (GSEA) was used to perform to investigate the biological pathway difference between high and low LINC01614 patients. The reference gene set was “Hallmark.v7.4.gmt”. The input data of GSEA analysis has been uploaded in figshare website (https://figshare.com/articles/dataset/GSEA/16746517).

Tissue and Cell Lines

The bladder cancer tissue and matched normal tissue were all collected from Taixing People’s Hospital. All the patients have signed a written informed consent form. The Ethics committee of Taixing People’s Hospital approved the protocol of this study before beginning. Four bladder cancer cell lines (5637, J82, RT4 and T24) and one normal bladder epithelial cell (SV-HUC-1) were purchased from the Cell Bank of Culture of the Chinese Academy of Sciences and routinely maintained in our lab with 37°C and 5% CO₂. This study was performed in accordance with the principles of the Declaration of Helsinki.

Quantitative Real-Time PCR (qRT-PCR)

RNA extraction was performed when cells were grown to a density of 80% confluence. In brief, total RNA was extracted using an RNA extraction kit (Invitrogen) and then reverse transcribed into cDNA. A Sybr Green PCR system was used to detect PCR amplicons. The primers used were as follows: LINC01614, forward primer, 5'-AGAGGCAAGTTCTAAAGG-3', reverse primer: 5'-AATGTGCGATAGTTCAGTC-3'; RUNX2, forward primer, 5'-TGGTTACTGTACATGGCGGTA-3', reverse primer, 5'-TCTCAGATCGTGAACCTTGCTA-3'.

Western Blotting

The protein extraction was conducted when cells were grown to a density of 100% confluence. In brief, cells were added with RIPA lysis buffer and then shocked on the ice for 2h. After frozen at −80°C overnight, the mixture was centrifuged 30 min at 4°C and the protein was drawn from the supernatant. The protein mixed with 1× loading buffer was loaded onto 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane for further exposure. The primary antibodies were purchased from Proteintech (GSK3B polyclonal antibody, phospho-Gsk3b polyclonal antibody, beta-catenin polyclonal antibody, C-MYC polyclonal antibody, beta actin polyclonal antibody).
Plasmid Construction and Cell Transfection

The LINC01614 knockdown, RUNX2 overexpressed and corresponding control plasmid were synthesized by Synthgene (China). Lipofectamine 3000 (Thermo Fisher, USA) was used for cell transfection following the manufacturer’s protocol. The shRNA sequence of LINC01614 for cell transfection were as follows: shRNA#1: 5’-CACATGACATAATCTGGGTTCTTTA-3’; shRNA#2: 5’-GGACAATGAAGACTGAACT-3’; shRNA#3: 5’-GGAC TTCAGACACGGAGAA-3’.

CCK8 Assay

Cells were resuspended and seeded into a 96-well plate. CCK8 assay was performed using a CCK8 kit (Dojindo, Shanghai, China). In brief, cells that were added with CCK8 reagent were then incubated for 2 hours. The absorbance of 450nm reflecting the cell proliferation activity was measured at 0, 24, 48 and 72h time points.

Colony Formation Assay

Cells were resuspended and seeded into a six-well plate with 500 cells per well. The medium was changed every 4 days. After that for 12 days, cells were fixed with formaldehyde and stained with crystal violet for counting.

Cell Apoptosis

Cell apoptosis was assessed by flow cytometry assay with Annexin V/propidium iodide (PI) staining. Cells were resuspended with 1× binding buffer. FACS Calibur cytometer (BD Biosciences) was then used to aspirate cells and perform analysis. FlowJo software was used for data processing.

5-Ethynyl-2-Deoxyuridine (EdU) Assay

An EdU labeling kit (Beyotime) was used to perform EdU assay to evaluate the DNA synthesis in cells. In brief, cells were incubated with EdU solution for 2 hours and then followed a set of steps according to the kit instructions. After stained with fluorescent and DAPI, cells were observed and photographed by fluorescence microscopy.

Transwell Assay

The transwell chambers separate the 24-well plate into the upper chamber and lower chamber. In brief, 200 µl serum-free conditioned medium mixed with 2×10⁶ cells. The lower chamber was added with a 600 µl medium containing 20% fetal bovine serum. After that for 24h, the chamber was removed. Cells were fixed with formaldehyde and stained with crystal violet for counting.

Wound Healing Assay

Wound healing assay was conducted when the cells were grown to a density of 100% confluence at a six-well plate. A 200-µl pipette tip was used to make scratches. Then, the cells were added with 1mL serum-free conditioned medium. The time points for observing were set as 0h and 24h.

In vivo Tumor Xenografts

Human T24 xenografts were established by subcutaneously inoculating 5×10⁶ cells resuspended in PBS into BALB/c nude mice (5–8 weeks of age). These mice were purchased from the Nanjing Animal Center and randomly divided into sh-NC and sh-LINC01614 group. On 7–8 weeks of age, all the mice were sacrificed, and tumors were excised.

Fluorescence in situ Hybridization (FISH)

The Cy3-labeled LINC01614 probe was designed and purchased from Jima Com (Shanghai, China). In brief, T24 cells were plated and incubated overnight. Then, the cells were fixed with 100 µl 4% paraformaldehyde for 15 min at room temperature. According to the protocol of RNA FISH probe kit purchased from Jima Com (Shanghai, China), the probes were diluted (1:50), denatured, balanced and added to cells at 37 °C overnight. Finally, cell nuclei were counterstained with DAPI–Antifade. LINC01614 FISH probe used was 5’-CTCCTTCCGACTTACTTGTC-3’.

Statistical Analysis

All the statistical analyses were performed using the R software, SPSS and GraphPad Prism 8. Student’s T-test was used for the data meeting the normal distribution, and Mann–Whitney U-test was used for the data meeting the non-normal distribution. P-value was two-sided and less than 0.05 was regarded as statistically significant.

Results

LINC01614 is Up-Regulated in Bladder Cancer and Associated with Poor Clinical Features

Firstly, we performed differential expression analysis with the threshold of |log2FC| >2 and adj.P.value <0.05 to identify differentially expressed IncRNAs between normal and bladder cancer tissue, in which 324 IncRNAs were down-regulated and 428 IncRNAs were up-regulated (Figure 1A).
To identify cancer-promoting lncRNAs, we refined the screening conditions with tumorMean >2 and HR >1. Finally, four lncRNAs AC099850.3, LINC01614, MNX1-AS1, AC006329.1 were identified (Figure 1B). Among these, the lncRNA LINC01614 has been reported to promote cancer progression in other cancers. However, it has not been mentioned in bladder cancer. Therefore, the lncRNA LINC01614 aroused our interest and was chosen for further research. Next, we comprehensively analyzed the LINC01614 expression level in the TCGA-BLCA project, including 19 normal and 414 tumor samples. The results showed a higher expression level of LINC01614 in all samples and 19 paired samples (Figure 1C and D). Also, we observed that LINC01614 was highly expressed in our own 16 paired bladder cancer tissue (Figure 1E). At cell level, LINC01614 was also elevated in four bladder cancer cell lines (5637, J82, RT4 and T24) compared with normal bladder epithelial cells (SV-HUC-1) (Figure 1F). Next, we explored the correlation between LINC01614 and clinical features, including age, gender, clinical stage, grade and prognosis. Interestingly, we found that older patients might have a higher LINC01614 expression, aligned to the morbidity pattern of bladder cancer (Figure 2A). However, no significant difference was observed in male and female patients (Figure 2B). To be noted, we found that the LINC01614 tends to be highly expressed in patients with worse clinical features (High grade vs Low grade; Stage III–IV vs I–II; T3-4 vs T1-2; N1-2 vs N0; M1 vs M0) (Figure 2C–G). Furthermore, Kaplan–Meier survival curves demonstrated that the patients with high LINC01614 expression had a shorter survival time compared with those with low LINC01614 expression (Figure 2H).

**LINC01614 Promotes the Proliferation of Bladder Cancer Cells**

To further explore the biological role of LINC01614 in bladder cancer, we performed cell transfection to knockdown endogenous LINC01614 expression, whose efficiency was validated by qRT-PCR (Figure 3A and B). CCK8 assay revealed that the cells with LINC01614 knockdown had a lower

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**Figure 1** LncRNA LINC01614 was up-regulated in bladder cancer tissue and cell lines.  
**Notes:** (A) Differential expression analysis with the threshold of logFC >2 and adj.P <0.05 to identify differentially expressed lncRNAs between normal and bladder cancer tissue; (B) intersection with the lncRNAs meeting logFC >2, adj.P <0.05, tumorMean >2 and HR >1 identified four lncRNAs; (C) LINC01614 was up-regulated in bladder cancer tissue (TCGA-BLCA, 19 normal tissue and 414 tumor tissue), ***p < 0.0001; (D) LINC01614 was up-regulated in paired bladder cancer tissue (TCGA-BLCA, 19 normal tissue and paired 19 tumor tissue), ****p < 0.0001; (E) LINC01614 was up-regulated in 16 paired bladder cancer tissue, ***p < 0.0001; (F) LINC01614 was up-regulated in bladder cancer cell lines, ****p < 0.0001, ****p < 0.0001.  
**Abbreviation:** TCGA, The Cancer Genome Atlas.
absorbance at 450nm, indicating that the inhibition of LINC01614 could hamper cell proliferation activity (Figure 3C and D). Meanwhile, colony formation assay showed that the cells in the knockdown interference group significantly decreased the colony number (Figure 3E). Another aspect, flow cytometry assay indicated that the inhibition of LINC01614 could remarkably increase the cell apoptosis rate (Figure 3F). Also, we found that tumor-forming ability in mice bearing with sh-LINC01614 was remarkably decreased compared with the control sh-NC T24 cells (Figure 3G).

**LIN01614 Facilitates Cell Metastatic Potential of Bladder Cancer Cells**

We further explore whether LINC01614 could promote cell invasion. Transwell assay showed that LINC01614 silencing could significantly inhibit cell invasion and migration ability of bladder cancer cells (Figure 4A and B). Meanwhile, the wound healing assay showed that the scratch width narrowed significantly in the LINC01614 knockdown group in 24h (Figure 4C).

**LINC01614 Affect the Activity of Wnt/β-Catenin Signaling Pathway**

GSEA analysis showed that the LINC01614 might be involved in the activation of Wnt/β-catenin signaling pathway (Figure 5A). Therefore, we performed Western blotting assay to explore the key molecular change of the Wnt/β-catenin signaling pathway (p-GSK-3β, GSK-3β, β-catenin and c-myc). The result demonstrated that the inhibition of LINC01614 could significantly decrease the protein level of p-GSK-3β, β-catenin and c-myc, suggesting that LINC01614 could upregulate the Wnt/β-catenin pathway activity (Figure 5B).

**RUNX2 is a Target Gene of LINC01614 and Positively Correlated with LINC01614**

Based on the expression profile data in TCGA, we screened the mRNA with a striking correlation with LINC01614 (|Cor > 0.3| and P < 0.01). In addition, we noticed that the gene RUNX2 reported promoting bladder cancer progression was significantly positively correlated with LINC01614 (Figure 6A and B, Cor = 0.52, P < 0.001). Moreover, we found that the patients with high RUNX2 expression might be associated with a worse prognosis and clinical features (Figure 6C–F). FISH assay showed that the LINC01614 was mainly localized in the cytoplasm, indicating that it may act through a competing endogenous RNA mechanism (Figure 6G). Furthermore, the intersection of Starbase and LncBase v2 identified two miRNA, miR-217 and miR-6807-3p, which...
might interact with LINC01614 (Figure 6H). qPCR results indicated that LINC01614 could regulate RUNX2 expression through miR-217, but not miR-6807-3p (Figure 6I–L).

**LINC01614 Acts as an Oncogene Effect in Bladder Cancer Through the RUNX2/Wnt/β-Catenin Axis**

We further overexpressed RUNX2 in LINC01614 knockdown cells to explore the underlying association between RUNX2 and LINC01614, whose transfection efficiency was validated by qRT-PCR (Figure 7A). CCK8 and colony formation assay showed that the inhibition of LINC01614 could significantly hamper the cell proliferation ability of bladder cancer cells, and this effect might be partly reversed by RUNX2 overexpression (Figure 7B and C). Transwell assay also indicated that the knockdown of LINC01614 remarkably reduced the invasion and migration cells, which was antagonized by overexpression of RUNX2 (Figure 7D). Same conclusion was also observed in the EdU assay that RUNX2 might reverse the anticancer effect brought by the knockdown of LINC01614 to some extent (Figure 7E). Considering the fact that we proved the Wnt/β-catenin signaling pathway to be the
downstream pathway of LINC01614, we therefore assessed the change of the Wnt/β-catenin molecular marker in sh-NC, sh-LINC01614 and sh-LINC01614 + RUNX2-OE cells. Western blotting demonstrated that the protein level of p-GSK-3β, β-catenin and c-myc were decreased in the LINC01614 knockdown cells, but rescued when the RUNX2 was overexpressed (Figure 7F–H).

**Discussion**

As the most common malignancy of the urinary tract, bladder cancer is a major cause of cancer-associated mortality worldwide.\(^\text{15}\) Although early diagnosis effectively improves patients’ survival, the disease heterogeneity of different stages worsen patients’ prognosis. Therefore, it is imperative to find a novel and effective tumor and prognosis biomarker for bladder cancer diagnosis and treatment.

With the advent of the human genome era, lncRNA has been widely reported to participate in the regulatory process of cell life, including metabolic regulation, epigenetics, molecular bridge, etc.\(^\text{16}\) Also, lncRNA plays a vital role in cancer tumorigenesis and development. For example, Logotheti et al revealed that the lncRNA SLC16A1-AS1 could promote bladder cancer progression by inducing metabolic reprogramming and co-activating E2F1.\(^\text{17}\) Ni et al identified a novel lncRNA uc.134 that was...
Figure 6 Identification of RUNX2 as a target gene of LINC01614.

Notes: (A) Identification of the genes significantly correlated with LINC01614 with the threshold of |Cor| > 0.3 and P < 0.01; (B) RUNX2 was positively correlated with LINC01614 (r = 0.52, P < 0.001); (C) Kaplan-Meier survival curve showed that the patients with high RUNX2 expression were associated with a worse prognosis; (D) the RUNX2 expression level in different clinical stage groups (stage I-II vs stage III-IV), ****P < 0.0001; (E) the RUNX2 expression level in different T stage groups (T1-2 vs T3-4), ****P < 0.0001; (F) the RUNX2 expression level in different N stage groups (N0 vs N1-2), ****P < 0.0001; (G) FISH assay showed that LINC01614 was mainly localized in the cytoplasm; (H) the intersection of Starbase and LncBase v2 identified two miRNAs, miR-217 and miR-6807-3p; (I-L) qPCR results indicated that LINC01614 could regulate RUNX2 expression through miR-217, but not miR-6807-3p. NS. P > 0.05, ***P < 0.001.

Abbreviation: FISH, fluorescence in situ hybridization.
Figure 7 LINC01614 promote bladder cancer progression through RUNX2/Wnt/β-catenin axis.

Notes: (A) The RUNX2 expression in sh-LINC01614 and sh-LINC01614+RUNX2-OE cells, ***P < 0.001; (B) CCK8 assay was performed in sh-NC, sh-LINC01614 and sh-LINC01614+RUNX2-OE cells, ***P < 0.001; (C) colony formation assay was performed in sh-NC, sh-LINC01614 and sh-LINC01614+RUNX2-OE cells, ***P < 0.001; (D) transwell assay was conducted in sh-NC, sh-LINC01614 and sh-LINC01614+RUNX2-OE cells, ***P < 0.001; (E) EdU assay was performed in the sh-NC, sh-LINC01614 and sh-LINC01614+RUNX2-OE cells, ***P < 0.001; (F–H) Western blot was used to detect the marker of Wnt/β-catenin pathway and RUNX2 in different groups, NS. P> 0.05, ***P < 0.001.
down-regulated in hepatocellular carcinoma.\textsuperscript{18} Meanwhile, they found that the lncRNA uc.134 could significantly hamper LAST1 degradation and ubiquitination mediated by CUL4A, thereby increasing Hippo kinase activity and inhibiting hepatocellular carcinoma progression.\textsuperscript{18} Marin-Béjar et al demonstrated that the lncRNA LINC-PINT was widely down-regulated in multiple cancer and had a highly conserved sequence element that was important for its function.\textsuperscript{19} More intensively, the conserved sequence mediates the interaction between lncRNA LINC-PINT and PRC2, which is essential for the repression of cancer-inhibiting signature of genes regulated by the transcription factor EGR1. In our study, the lncRNA LINC01614 was first identified to promote bladder cancer progression, which has not been previously reported. Our result showed that the lncRNA LINC01614 could be an underlying tumor and prognosis biomarker of bladder cancer.

Based on the expression profile data of TCGA, we identified the gene RUNX2 as a downstream gene of LINC01614. RUNX2 is a member of the RUNX family of transcription factors and encodes a nuclear protein with a runt DNA-binding domain.\textsuperscript{20} The aberrant expression pattern of RUNX2 has been observed in most solid tumors, indicating its close genetic association with initiation and progression.\textsuperscript{21,22} In papillary thyroid carcinoma, Sancisi et al found that the RUNX2 was controlled by Id1 and activated a gene list involved in the matrix degradation and cell invasion to facilitate cancer progression.\textsuperscript{23} In pancreatic cancer, Kayed et al indicated that TGF-beta1 and BMP2 regulated RUNX2 in an auto- and paracrine manner to affect tumor microenvironment, thereby promoting cancer progression.\textsuperscript{24} Moreover, a 5-year follow-up study showed that lung cancer patients with higher RUNX2 mRNA expression were associated with a worse prognosis.\textsuperscript{25} Meanwhile, multiple studies demonstrated that RUNX2 acts as an oncogene in bladder cancer through different genetic mechanisms, indicating its diverse effect patterns.\textsuperscript{14,26} Our result showed that LINC01614 positively regulated RUNX2 expression and partly dependent on RUNX2 to promote cancer progression, refining the function network of RUNX2 in bladder cancer.

Wnt/β-catenin signaling pathway is a critically important axis that affects the myriad biological processes throughout animal development.\textsuperscript{27} In parallel, abnormal Wnt/β-catenin activity was responsible for many pathological processes, including cancer initiation and progress.\textsuperscript{27} Yang et al indicated that LINC01133 regulated the activity of Wnt/β-catenin signaling through a competitive endogenous RNA (ceRNA) mechanism to inhibit gastric cancer progression.\textsuperscript{28} In breast cancer, Li et al showed that c-myc could interact with β-catenin and activate the Wnt/β-catenin downstream genes Cyclin D1 and Axin2 to influence breast cancer progression.\textsuperscript{29} Furthermore, accumulating evidence has strengthened the potential of anti-cancer drugs targeting the Wnt/β-catenin signaling pathway, for example, PORCN and FZD inhibitor.\textsuperscript{30} Our results proved that the LINC01614 might activate the Wnt/β-catenin pathway activity, indicating its potential to be a target for bladder cancer targeted therapy.

To the best of our knowledge, this study is the first study focused on the role of LINC01614 in bladder cancer. Here, we found that LINC01614 was up-regulated in the bladder cancer tissue and cell lines. Meanwhile, the high expression level of LINC01614 was associated with poor clinical features and a shorter survival time. In vitro experiments demonstrated that LINC01614 could remarkably facilitate bladder cancer proliferation and invasion. RUNX2 was then found positively correlated with LIN01614 in RNA level. Further experiments showed that the overexpression of RUNX2 could partly rescue the cancer-inhibiting effect brought by the LINC01614 knockdown. GSEA analysis and Western blot assay proved that the Wnt/β-catenin was the downstream pathway of LINC01614. Overall, our result showed that LINC01614 promotes bladder cancer progression through the RUNX2/Wnt/β-catenin axis, making it an underlying tumor biomarker and therapeutic target.

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**Disclosure**

The authors report no conflicts of interest in this work.

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