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LncRNA LINC00885 promotes bladder cancer progression by targeting the miR-98-5p/PBX3 axis

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ABSTRACT

Depicted as the most prevalent malignancy, bladder cancer (BLCA) associated deaths in males all around the world. Increasing evidence has uncovered that dysregulation of lncRNA is associated with the complex processes of various tumors. Although recent research concerning bladder cancer has mentioned the participation of lncRNALINC00885, the specific regulatory role of LINC00885 in BLCA hasn't been elucidated. This study aimed to explore the regulatory role of LINC00885 in BLCA. For this purpose, qRT-PCR checked the LINC00885 expression. CCK-8, caspase-3, colony formation, and western blot (WB) experiments were carried out to intestate LINC00885 specific role in BLCA. RIP and RNA pull-down assays were used to study the regulation effect between miR-98-5p and LINC00885 (or PBX3) in BLCA. Results showed that LINC00885 was up-regulated in BLCA and promoted cell proliferation, inhibited cell apoptosis in BLCA. Molecular mechanism experiments displayed that miR-98-5p could bind to LINC00885 and PBX3. Up-regulated miR-98-5p reduced cell proliferation, and facilitated cell apoptosis in BLCA. Besides, miR-98-5p could down-regulated PBX3 expression while LINC0088 could up-regulate PBX3 in BLCA. Final rescue tests demonstrated that PBX3 deficiency reversed the miR-98-5p inhibition effect on the progression of sh-LINC00885#1-transfected cells. In conclusion, LINC00885 enhances BLCA progression by targeting the miR-98-5p/PBX3 axis, revealing that LINC00885 might serve as a novel molecular marker in bladder cancer treatment.

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Introduction

Accepted as a common disease in the genitourinary system worldwide, bladder cancer (BLCA) has high incidence and mortality rates causing cancer-associated deaths in males around the world (1-3). Despite efforts made in BLCA therapies, including the prevalent employment of minimally invasive surgery and a better comprehension of multimodal treatments (chemotherapy, radiotherapy as well as immunotherapy), the outcome is still unsatisfying (4-6). Therefore, further exploration of molecular mechanisms remains to be urgent for the purpose of identifying a therapeutic target for BLCA.

Noncoding RNAs (ncRNAs), not like the traditional coding genes which elicit their biological impact when translated into protein molecules, exert critical regulatory function on cancer progression in form of RNA and have no coding capacity (7). Long noncoding RNA (lncRNA) is a member of ncRNAs, with a vital regulatory role in human cancers (8). LncRNA RP4 sponges miR-7-5p in colorectal cancer (9). LncRNA LUCAT1 enhances ovarian cancer progression viamiR-612/HOXA13 axis (10). LncRNA MALAT1 inhibits cell metastasis in breast cancer (11). Additionally, an existing body of literature has confirmed the molecular mechanisms of different lncRNAs in BLCA. LncRNA ZFAS1 promotes the tumorigenesis of BLCA by competitively binding with miR-329 (12). LncRNA FGFR3-AS1 is involved in BLCA cell proliferation and apoptosis (13). LncRNA ATB accelerates BLCA cell proliferation and metastasis by suppressing miR-126 (14). LncRNA GHET1 accelerates the progression of BLCA (15). LINC00885, identified as a lncRNA recently, has been mentioned in a previous investigation concerning BLCA, but not its specific function (16,17). Thus, the underlying role of LINC00885 in BLCA remains to uncover.

Here, we were dedicated to probing the function and molecular mechanism of LINC00885 in BLCA. The results of this study testified that LINC00885 promotes BLCA progression via the miR-98-5p/PBX3 axis, which to a certain degree sheds new light on BLCA researches.

Materials and Methods

Cell culture

Human BLCA cell lines (5637, SW780, UM-UC-3, T24) and normal bladder epithelial cell line (SV-HUC-1) were acquired from CASC (Shanghai, China), and were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% FBS (Gibco) and antibiotics with 5% CO_2 at 37°C.

Plasmid transfection

Lipofectamine™3000 (Thermo Fisher Scientific, USA)

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was used to cell transfection in 12-well plates for 48 h as per the user manual. Two specific shRNAs to LINC00885 and PBX3 (sh-LINC00885#1/2 andsh-PBX3#1/2) were obtained from RiboBio Corporation (Guangzhou, China), along with control shRNAs (sh-NC). MiR-98-5p mimics, miR-98-5p inhibitor and their respective controls (NC mimics/inhibitor) from GenePharma (Suzhou, China) were utilized in this study.

RT-qPCR analysis

Total RNA extraction was acquired with TRIzol (Invitrogen, Karlsruhe, Germany). Quantification was conducted by a NanoDrop ND-2000 (Thermo Fisher Scientific). Synthetic cDNA was subjected to a SYBR Green PCR kit (Takara, Tokyo, Japan) for qPCR. The average value was calculated by the comparative $2^{-\Delta\Delta Ct}$ method. House-keeping gene GAPDH or snRNA U6 was taken as the loading control.

CCK-8

Cells were cultured in 96-well culture plates for 24 h after transfection. Following the addition of CCK-8 (Beyotime, China), the absorbance was estimated per well at 0, 24, 48, 72 and 96 h by a microplate reader (Bio-Rad, USA).

Colony formation

Transfected cells in 6-well plates were cultivated for 2 weeks. The fixed colonies containing were stained by the use of crystal violet solution (0.1%). At last, colonies were counted and captured by a microscope.

Caspase-3 activity

Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) determined caspase-3 activity. Reaped cells, were stained in lysis buffer for 15 min at 4 °C, centrifugated and collected supernatant. Ac-DEVD-pNA was incubated with supernatant for 2 h at 37°C. The final absorbance (405 nm) was measured with a microplate reader.

Western blotting

Reagent radioimmunoprecipitation assay (RIPA; Beyotime Institute of Biotechnology) was applied for total cellular proteins. Following quantification with a BCA kit (TaKaRa, Japan), the protein was separated by SDS-PAGE. Transferred to PVDF membranes (Millipore, USA). Incubated primary/secondary antibody bought by Abcam (Cambridge, USA). Antibodies against Bcl-2(ab196495), Bax (ab53154), PBX3 (ab56239) and GAPDH (ab110305) were utilized. At length, assessed the protein expression with ELC.

Subcellular fractionation

RNAs were separated with SurePrep Nuclear or Cytoplasmic RNA Purification Kit (Thermo Fisher Scientific) following the guidebook. U6 snRNA or GAPDH mRNA was examined in extracted RNAs as a control for nuclear or cytoplasm RNA.

RNA pull-down

Cells were treated with biotinylated probes against LINC00885 or miR-98-5p (LINC00885 biotin probe, miR-98-5p biotin probe; Thermo Fisher Scientific). Following the addition of streptavidin magnetic beads, the complexes were eluted and isolated for RT-qPCR.

Luciferase reporter assay

The wile-type luciferase reporter vectors LINC00885-WT and PBX3 WT were generated by inserting the predictive miR-98-5p binding site ofLINC00885 sequences and 3'-UTR of PBX3into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA). miR-98-5p binding sites mutations were utilized to construct mutant vectors and termed as LINC00885-Mut and PBX3 MUT-1/2. Cells were co-transfected with LINC00885 vectors or PBX3 vectors and miR-98-5p mimics or NC mimics by use of Lipofectamine™3000 for 48 h. The result was utilized after transfection as the supplier requested.

Microarray profiling

Total RNAs were extracted from transfected cells 48 h post-transfection and PCR amplified. By use of Low-input Quick Amp Labeling kit One-color (Agilent Technologies, USA), synthetic cRNA was hybridized and scanned on the Agilent Microarray Scanner. Microarray data were detached by GeneSpring GX version 13 (Agilent Technologies). In accordance with the cut-off criteria of $p<0.05$ and fold-change >2, differentially expressed mRNAs were screened out.

RNA immunoprecipitation (RIP)

RIP assay was performed utilizing RNA Immunoprecipitation Kit (Millipore). The collected cells were kept in a lysis buffer and centrifuged. Cell supernatant was added with protein-A/G-Sepharose beads and antibodies of interest at 4°C all night. The retrieved RNAs were collected for RT-qPCR.

Statistical analysis

Data of at least three biological replications were shown as mean ± SD. Differences between groups were assayed by two-tailed Student's t-tests or one-way ANOVA applying SPSS Statistics 20.0 software (IBM, Armonk, NY, USA). The threshold of statistical significance was set as p-value < 0.05.

Results

LINC00885 is highly expressed and its depletion suppresses BLCA cell proliferation and promotes apoptosis

Existing literature has mentioned the participation of LINC00885(16), but not the specific regulatory role of LINC00885 in BLCA. First, we detached the expression level of LINC00885 in tissues and cells of BLCA. As displayed in Figure 1A, LINC00885 was up-regulated in BLCA tissues according to the TCGA database. RT-qPCR analysis uncovered a notable upregulation of LINC00885 in BLCA cell lines (5637, SW780, UM-UC-3 and T24) (Figure 1B).

To ensure the LINC00885 role, an RT-qPCR assay was performed to ensure it knockdown efficiency. After knocking down LINC00885 by use of sh-LINC00885#1 or sh-LINC00885#2 in T24 and UM-UC-3 cells, LINC00885 expression was markedly reduced (Figure 1C). Subsequently, cell proliferation ability was checked utilizing CCK-8 and colony formation analysis. The results revealed that LINC00885 deficiency restrained the proliferation

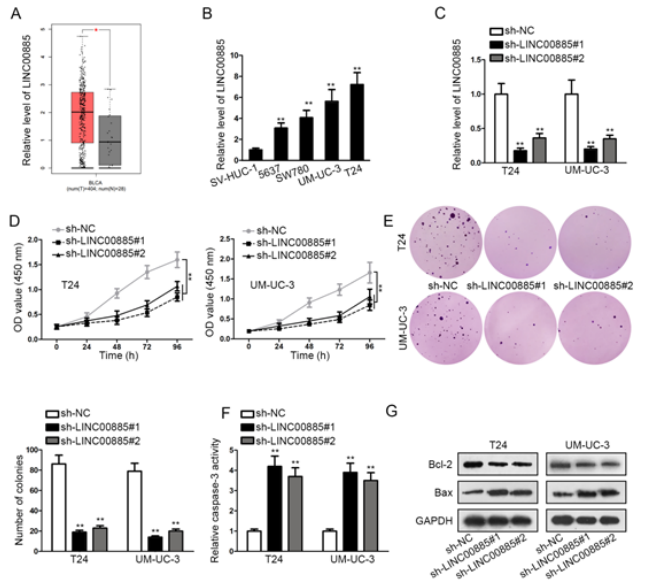

Figure 1. LINC00885 is highly expressed and its depletion suppresses cell proliferation whereas promotes cell apoptosis in BLCA. (A) The expression of LINC00885 in BLCA tissues and adjacent non-tumor tissues was revealed through the TCGA database. (B) The expression of LINC00885 in BLCA cell lines (5637, SW780, UM-UC-3 and T24) and human normal bladder epithelial cell line (SV-HUC-1) was detected by RT-qPCR. (C) RT-qPCR assay was utilized to examine the knockdown efficiency of LINC00885 in T24 and UM-UC-3 cells. (D-E) The proliferation ability of transfected cells was measured by CCK-8 and colony formation assays. (F) Caspase-3 activity analysis examined the apoptosis capability of T24 and UM-UC-3 cells transfected with sh-LINC00885#1/#2 or sh-NC. (G) The expression of Bcl-2 and Bax in transfected cells was detected by WB assay. $^{**}P<0.01$.

of BLCA cells (Figure 1D-E). Furthermore, the apoptosis capability of UM-UC-3 and T24 cells was enhanced by LINC00885 depletion based on the analysis of caspase-3 activity (Figure 1F). In addition, a notably downregulated of Bcl-2 and an evidently upregulated of Bax were detected by WB assay after BLCA cells were transfected with sh-LINC00885#1/2, indicating that LINC00885 silencing induced cell apoptosis (Figure 1G). To sum up, LINC00885 is overexpressed in BLCA, LINC00885 knockdown represses BLCA cell proliferation and promotes apoptosis.

LINC00885 sponges miR-98-5p and miR-98-5p overexpression inhibits BLCA cell proliferation and facilitates cell apoptosis

To cover the LINC00885 molecular mechanism in BLCA, we first detect the distribution of LINC00885 in the cytoplasm and nucleus. Subcellular fractionation analysis suggested a major proportion of LINC00885 in the cytoplasm (Figure 2A). Hence, finding the specific miRNA that LINC00885 sponged to facilitate bladder cancer progression was urgently needed at present. Through searching starBase, 13 miRNAs (miR-4458, let-7i-5p, miR-4500, let-7g-5p, let-7f-5p, miR-98-5p, let-7c-5p, miR-654-3p, let-7b-5p, miR-3613-5p, let-7a-5p, let-7d-5p, and let-7e-5p) that have the bind ability with LINC00885were listed in Figure 2B. Then RNA pull-down demonstrated that miR-98-5p was the most observably enriched in the LINC00885 biotin probe group (Figure 2C). Next, bioinformatics analysis indicated LINC00885 could bind tomiR-98-5p (Figure 2D). Later, pmirGLO-LINC00885-WT luciferase activity was evidently reduced by miR-98-5p mimics in BLCA cells (Figure 2E). Afterward, an obvious lower expression of miR-98-5p in BLCA cell lines was checked by RT-qPCR (Figure 2F). Next, the miR-98-5p biological function in BLCA was probed. CCK-8 and colony formation assays certified that miR-98-5p overexpression reduced proliferation ability (Figure 2G-H) and promoted caspase-3 activity (Figure 2I). Additionally, the WB assay demonstrated a lowered Bcl-2 and an increased Bax in transfected miR-98-5p mimics T24 and UM-UC-3 cells, indicating that miR-98-5p overexpression accelerated cell apoptosis (Figure 2J). Taken together, LINC00885 sponges miR-98-5p, miR-98-5p overexpression inhibits BLCA cell proliferation and facilitates cell apoptosis.

PBX3 as a target gene of miR-98-5p in BLCA

Since growing evidence has indicated that miRNA modulates multiple tumors growth by direct regulation of its target gene (18,19), it was of great significance to ensure the miR-98-5p specific target gene in BLCA. First

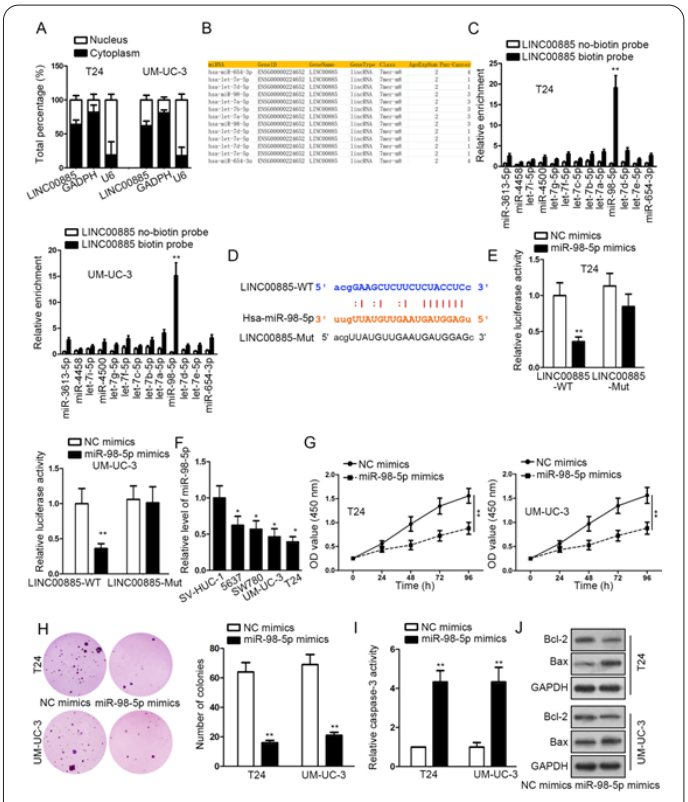

Figure 2. LINC00885 sponges miR-98-5p and overexpression of miR-98-5p inhibits cell proliferation but facilitates cell apoptosis in BLCA. (A) Subcellular fractionation assay detected the distribution of LINC00885 in the cytoplasm and nucleus. (B) MiRNAs that have the binding ability with LINC00885 were found by searching starBase. (C) RNA pull-down analysis demonstrated the enrichment of miRNAs in different groups. (D) Bioinformatics predicted that there existed a binding site between LINC00885 and miR-98-5p. (E) The interaction between LINC00885 and miR-98-5pin T24 and UM-UC-3 cells was confirmed by luciferase reporter assay. (F) The expression of miR-98-5p in bladder cancer cell lines and human normal bladder epithelial cell lines was detected by RT-qPCR. (G-H) CCK-8 and colony formation assays examined cell proliferation ability in transfected cells. (I) Cell apoptosis capability was analyzed by caspase-3 activity assay in transfected cells. (J) WB assay evaluated the expression of apoptosis-related proteins (Bcl-2 and BaX) in transfected cells. $^{*}P<0.05$, $^{**}P<0.01$.

of all, 14 candidate target genes (PPP1R15B, MDM4, YOD1, FIGN, PCGF3, MFSD8, CPA4, MTUS1, NR6A1, PBX3, RDX, IGDCC4, CDC34 and OPA3) of miR-98-5p was predicted by miRWalk database (Figure 3A). Then, the heat map displayed that PBX3 was downregulated obviously by miR-98-5p mimics (Figure 3B). Therefore, PBX3 was chosen to be further studied. WB assay depicted that PBX3 was dramatically cut down by overexpression of miR-98-5p (Figure 3C). After that, RT-qPCR and WB analyses were applied, and thus obtained a significant PBX3 mRNA and protein upregulated in the BLCA cell line (Figure 3D). Besides, a remarkably lowered PBX3 caused by LINC00885 knockdown was perceived in BLCA cells (Figure 3E). Furtherly, RIP analysis suggested that LINC00885, miR-98-5p and PBX3 bound to RNA-induced silencing complex (RISC) (Figure 3F). To further testify whether miR-98-5p could bind with PBX3, starBase was utilized to discover that there were two binding sites between miR-98-5p and PBX3, which was subsequently verified by luciferase reporter assay (Figure 3G-H). The final assay validated that miR-98-5p could bind with LINC00885 (or PBX3) in BLCA cells (Figure 3I). In a word, PBX3 is a miR-98-5p target gene in BLCA.

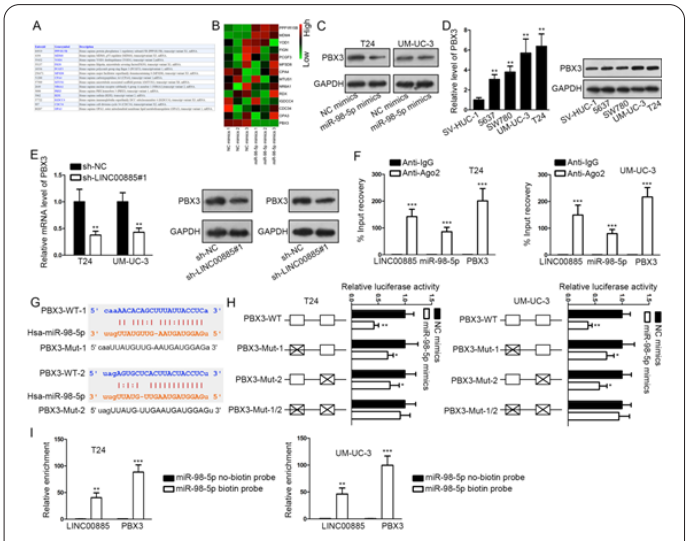

Figure 3. PBX3 functions as a target gene of miR-98-5p in bladder cancer. (A) MiRWalk database predicted 14 candidate target genes of miR-98-5p. (B) The expression of PBX3 in transfected cells was shown by a heat map. (C) The protein expression of PBX3 in transfected cells was detected by WB assay. (D) The mRNA and protein expression of PBX3 in bladder cancer cell lines and human normal bladder epithelial cell lines was detected by RT-qPCR and WB assays. (E) The mRNA and protein expression of PBX3 was detected by RT-qPCR and WB assays T24 and UM-UC-3 cells by transfection with sh-LINC00885#1 or sh-NC. (F) RIP analysis confirmed that LINC00885, miR-98-5p and PBX3 could bind to RISC. (G-H) PBX3 was found to have two binding sites for miR-98-5p, which was further tested by luciferase reporter assay. (I) The interaction between miR-98-5p and LINC00885 (or PBX3) was confirmed by RNA pull-down assay in T24 and UM-UC-3 cells. *P<0.05, **P<0.01, ***P<0.001.

LINC00885 promotes BLCA progression via miR-98-5p/PBX3 axis

Precious studies have suggested that lncRNA could act as a ceRNA in contributing to cell growth and metastasis in diverse cancers (9,20,21). In order to test whether LINC00885 promotes BLCA progression via the miR-98-5p/PBX3 axis, rescue experiments were employed in this study. Before conducting rescue assays, the PBX3 knockdown and miR-98-5p inhibition efficiency were assessed by RT-qPCR (Figure 4A). Thereafter, CCK-8 and colony formation assays certified that PBX3 knockdown rescued the miR-98-5p inhibition resulting in promoted function on cell proliferation in sh-LINC00885#1-transfected cells (Figure 4B-C). Furthermore, caspase-3 activity analysis indicated that the apoptosis capability of BLCA cells transfected with sh-LINC00885#1 was inhibited by miR-98-5p suppression, which was then reversed by silencing PBX3(Figure 4D). WB assay demonstrated that PBX3 downregulation countervailed the miR-98-5p inhibitor function on Bcl-2and Bax expression in sh-LINC00885#1-transfected cells, indicating that PBX3 depletion could reverse the miR-98-5p inhibition-regulated effect onBLCA cell apoptosis (Figure 4E). In summary, LINC00885 promotes BLCA progression via the miR-98-5p/PBX3 axis.

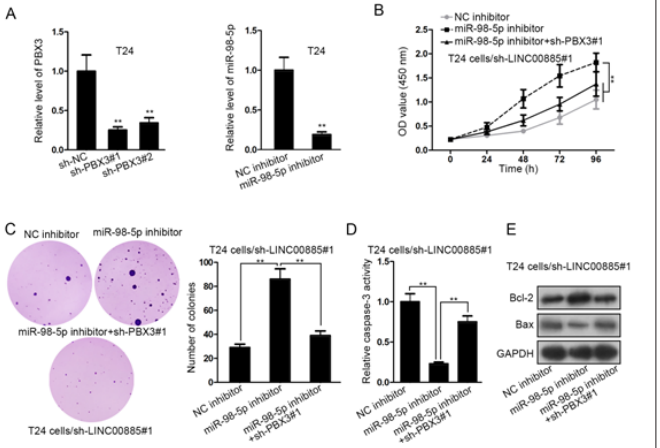

Figure 3. LINC00885 promotes BLCA progression via the miR-98-5p/PBX3 axis. (A) The knockdown efficiency of PBX3 and inhibition efficiency of miR-98-5p were assessed by RT-qPCR. (B-C) CCK-8 and colony formation assays analyzed the proliferation ability of transfected cells. (D) Caspase-3 activity analysis evaluated the apoptosis capability of transfected cells. (E) The expression of apoptosis-related proteins (Bcl-2 and Bax) in transfected cells was measured by western blot assay. **P<0.01.

Discussion

BLCA has been reported to be a common factor leading to cancer-associated deaths in men worldwide (13). Former studies have elucidated that lncRNAs participate in colorectal cancer (9), breast cancer (11) and bladder cancer (15) development. Although LINC00885 has been mentioned in a previous investigation concerning bladder cancer (16), its specific function remains unclear. We found that LINC00885 was highly expressed in BLCA tissues and cells. LINC00885 knockdown suppressed BLCA cell proliferation and promoted apoptosis. In brief, LINC00885 contributes to BLCA progression.

MicroRNAs (miRNAs), a subtype of ncRNAs, are associated with gene posttranscriptional regulation (22). It has been revealed that some biological processes of tumors were linked to the regulation of miRNAs. For illustration, the down-regulation of miRNA-143 promotes pancreatic cancer progression (23). MiRNA-21 and miRNA Let-7 are involved in breast cancer development (24). MiRNA-148a represses lung cancer cell metastasis through Wnt1

(25). MiR-98-5p has also been studied in some cancers, including osteosarcoma (26) and prostate cancer (27). Nonetheless, its role in interacting with LINC00885 in BLCA hasn't been clarified. In this study, LINC00885 was confirmed to bind tomiR-98-5p in BLCA.MiR-98-5pwas-low expressed in BLCA cells, and overexpression of it suppressed BLCA cell proliferation and promoted apoptosis. In other words, miR-98-5p as a tumor suppressor in the progression of BLCA, which could be sponged by LINC00885.

Owing to the fact that lncRNA can modulate tumor growth by competitively binding with miRNA to regulate mRNA expression in various cancers (9,20,21), we utilized the miRWalk database to obtain the candidate miR-98-5p target genes. Among these target genes, PBX3 was selected to be the object of this research because of its evident interaction with miR-98-5p and its oncogenic function on hepatocellular carcinoma (28), colorectal cancer (29) and acute myeloid leukemia (30, 31). This study tested that PBX3 was noticeably upregulated in BLCA. Besides, PBX3 was down-regulated by miR-98-5p whereas up-regulated by LINC00885 in BLCA. Moreover, miR-98-5p was validated to bind with LINC00885 (or PBX3) in BLCA. Final rescue assays further testified that PBX3 knockdown could rescue the miR-98-5p inhibition-mediated function on BLCA cell proliferation and apoptosis in sh-LINC00885#1-transfected cells.

Our data indicated that LINC00885 promotes BLCA progression via the miR-98-5p/PBX3 axis, which may be applied as an effective target for BLCA research.

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We appreciate all members.

Conflicts of interest
This study has no conflicts of interest.

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