LASP2 suppressed malignancy and Wnt/β-catenin signaling pathway activation in bladder cancer

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Abstract. LIM and SH3 Protein 2 (LASP2), a member of the nebulin family of actin-binding proteins, is associated with the development of cancers. However, little was known about the role of LASP2 in human bladder cancer. In the current study, LASP2 expression was evaluated by reverse transcription-quantitative polymerase chain reaction analysis in bladder cancer cell lines and tissue samples. The role of LASP2 in cancer cell proliferation, migration and invasion, and angiogenesis was explored. The association between prognostic outcomes and LASP2 expression were examined using Kaplan-Meier analysis. LASP2 expression was decreased in bladder cancer cells and tissues. LASP2 expression was associated with tumor size (P=0.016) and T classification (P=0.001). Patients with lower LASP2 expression had shorter overall and recurrent-free survival times. Overexpression of LASP2 inhibited and silencing of LASP2 promoted the proliferation, migration and invasion of bladder cancer cells, and angiogenesis in bladder cancer. Furthermore, it was determined that the tumor suppressing effect of LASP2 may be associated with the inactivation of the Wnt/β-catenin signaling pathway. LASP2 may represent a novel and useful prognostic indicator, and could serve as a potential therapeutic target for bladder cancer.

Introduction

Bladder cancer is one of the most common urogenital cancers worldwide and the 7th leading cause of cancer-associated mortality (1). Approximately 79,000 new cases are diagnosed each year in the USA, of which there are an estimated 16,800 mortalities (2). During the last several decades, important advances have occurred in the diagnosis and treatment of bladder cancer, which have led to promising and significant improvements clinically; however the survival rate of patients with late-stage disease remained unfavorable (3). Additionally, the underlying mechanisms of bladder cancer progression are largely unknown (4). Therefore, studies have focused on the molecular mechanisms of bladder cancer development and the identification of novel molecular biomarkers (5,6).

LIM and SH3 Protein 2 (LASP2) is a member of the nebulin family of actin-binding proteins characterized by a LIM motif and a Src homology region 3 domain (7). LASP2 was originally identified in chicken brains; the primary structure of LASP2 possesses a high degree of similarity with another nebulin family member, LASP1 (8). Like LASP1, LASP2 contains an N-terminal LIM domain, C-terminal SH3 domains and internal nebulin repeats (8). LASP1 and LASP2 are components of focal adhesions, and bind F-actin and zyxin (9). However, the sequence between the second nebulin repeat and the SH3 domain of LASP2 is markedly different from the sequence in the same area in LASP1 (8). The function of LASP2 has been linked to its LIM and SH3 domains. The LIM domain has been identified in diverse cellular functions in cell adhesion and signal transduction (10). The SH3 domain is characterized as a conserved sequence in the viral adaptor protein v-Crk, which is associated with the dynamics of the actin cytoskeleton (11). Therefore, the role of LASP-2 has been focused on focal adhesion function and organization. For example, Deng et al (12) revealed that LASP2 could increase the rate of attachment and migration of fibroblasts on fibronectin-coated surfaces, and may serve a role in cell proliferation. Recently, attention has focused on the function of LASP2 in tumorigenesis and tumor development (13,14). However, the role of LASP2 in bladder cancer has not yet been elucidated.

In the present study, it was revealed that LASP2 was down-regulated in bladder cancer, and that LASP2 expression was associated with the clinicopathological features of patients with bladder cancer. Overexpression of LASP2 effectively suppressed, while LASP2 depletion promoted the proliferation, migration and invasion of bladder cancer cells, and angiogenesis in bladder cancer. Furthermore, it was demonstrated that the Wnt/β-catenin signaling pathway is associated with the tumor-suppressing effects of LASP2 in bladder cancer cells. Therefore, the data from the present study highlighted the biological role of LASP2 in bladder cancer progression, and suggested that it has potential value as a therapeutic target for this disease.
Materials and methods

**Cell lines.** The bladder cancer cell line EJ (cat. no. CL-0274) was obtained from Procell Life Science and Technology Co., Ltd. (Wuhan, China), and 5637 (cat. no. TCHu 1), UM-UC-3 (cat. no. TCHu 217), T24 (cat. no. SCSP-536) and TCCSUP (cat. no. SCSP-571) bladder cancer cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained as described previously (15). Normal urothelial cells (provided by Huazhong University of Science and Technology, Wuhan, China) were obtained from fresh human bladder samples. All the cancer cell lines were cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO₂ atmosphere.

**Tissue samples.** A total of 196 bladder cancer RNA samples, which were retrieved from the archives of The First People’s Hospital of Jingmen (Jingmen, China), were used for reverse transcription–quantitative polymerase chain reaction (RT-qPCR) analysis. A total of 60 pairs of snap-frozen bladder cancer and normal adjacent-noncancerous tissues (48 male and 12 female, aged from 43-70 years) for RT-qPCR analysis were obtained from The First People's Hospital of Jingmen between January 2016 and February 2017. All patients were pathologically staged according to the American Joint Committee on Cancer's classification system on TNM staging (16). For the use of these clinical materials for research purposes, informed consent from all patients and approval from the Institutional Research Ethics Committee of The First People’s Hospital of Jingmen were obtained.

**Western blotting analysis.** Western blotting was performed according to standard methods as previously described (14). Cells and tissues were lysed with radioimmunoprecipitation assay lysis buffer (Biyuntian Biotechnology Research Institute, Nantong, China). The protein concentration of each lysate was determined with the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Finally, DAPI (1 µg/ml; Sigma-Aldrich; Merck KGaA) was added to the slide, and stained for 20 min at room temperature. Images were captured via fluorescence microscopy (magnification, x20). ImageJ 1.47 (National Institutes of Health, Bethesda, MD, USA) was used to analyze the resulting images.

**Labeling with 5-ethynyl-2'-deoxyuridine (EdU).** Cell proliferation assays were carried out using EdU labeling. Cells (1x10⁵/well) were plated in 96-well plates and cultured in RPMI 1640 medium. At 70-80% confluence, cells were incubated with 50 µM EdU (cat. no. R1053; Guangzhou RiboBio Co., Ltd., Guangzhou, China) for 3 h at 37°C and treated with 100 µl 1X ApolloR reaction cocktail (Guangzhou RiboBio Co., Ltd., Guangzhou, China) for 3 h at 37°C. The resulting samples were analyzed using Apollo Fluorometer (ThermoFisher Scientific, Inc.) according to the manufacturer’s protocol. Knockdown was assessed by RT-qPCR after 48 h of transfection.

**Transwell cell migration assay.** The migration of cells was assessed using 24-well Transwell chambers with 8-µm pore membranes (Corning Life Sciences, Corning, NY, USA). A total of 200 µl cell medium (1x10⁵ cells/100 µl in RPMI 1640 medium) were added to the upper chamber of each
Transwell chamber. Medium containing 10% FBS (HyClone; GE Healthcare Life Sciences) was added to the lower chamber. After incubating the cells for 24 h, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at 0˚C for 20 min and stained with 0.1% crystal violet for 15 min at 25˚C for visualization using a light microscope (magnification, x20). Migrated cells were measured by image analysis software (ImagePro Plus 6.0; Media Cybernetics, Inc., Rockville, MD, USA). Experiments were performed in triplicate. For cell invasion experiments, Matrigel (BD Biosciences) was plated inside Transwell culture inserts, with the same protocol being followed.

Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences.

| Gene     | Forward primer (5'-3')       | Reverse primer (5'-3')                              |
|----------|------------------------------|-----------------------------------------------------|
| LASP2    | CATTCCCAAGGCTATGGCTA         | ATCGTACATGGCTCGGTAGG                                 |
| c-Myc    | GGCCTCCTGGCAAAAGGTCA         | CTGCGTATGTTGTGCTGATGT                                 |
| c-Jun    | TCCAAGTGCCGAAAAAGGAAG        | CGAGTTCTGAGCTTTCAAGGTT                               |
| CCND1    | CAATGACCCCCGCACGATTTC        | CATGGAGGCGGGATTTGAA                                  |
| CTLA4    | CATGATGGGGGAATGAGTTGACCC     | ATCTGCTCTGAGATAGGTAGGTTC                             |
| LEF1     | ATGTCACATCCAAACAAGGCA        | CCGGAGCAAGGGATAAAAAAGT                               |
| TCF1     | TTTAGTCTAGTCTGTTGTACC        | CTTTGGAAGCTCTGCTTTGAGC                              |
| AXIN2    | ACTGCCACACACAGTAAGGAG        | CTGGCTATGCTTTGGAACCA                                 |
| GAPDH    | TCAAGGAAGTGGTTGAAGCAG        | CTGCAAAAGTGAGGATTG                                   |

LASP2, LIM and SH3 Protein 2; CCND1, cyclin D1; CTLA4, cytotoxic T lymphocyte associated protein 4; LEF1, lymphoid enhancer binding factor 1; TCF1, T cell factor 1.

Figure 1. LASP2 expression is decreased in patients with bladder cancer. (A) LASP2 mRNA expression in 60 pairs of bladder cancer and adjacent non-tumor tissues. (B) LASP2 mRNA expression in NUCs and bladder cancer cell lines (EJ, 5637, UM-UC-3, T24 and TCCSUP). *P<0.05 vs NUC. (C) Kaplan-Meier analysis of overall and recurrent-free survival rates stratified by low and high LASP2 expression (n=98/group). LASP2, LIM and SH3 Protein 2; NUC, normal urothelial cell; T/N, tumor/normal.
Human umbilical vein endothelial cells (HUVECs) tube formation assay. Precooled Matrigel (Corning Life Sciences) was plated into each well of a 24-well plate and allowed to set into a gel for 30 min at 37°C. HUVECs (5x10^4/well; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) in 200 µl F-12K medium (Hyclone; GE Healthcare Life Sciences) were added to each well and incubated at 37°C in 5% CO_2 for 20 h. The capillary tube structure was photographed using a light microscope (magnification, x20) and quantified by measuring the total length of the completed tubes. ImageJ 1.47 was used to analyze the resulting images.

Luciferase reporter assay. Cells (1x10^5 cells/well) were seeded in 96-well plates and allowed to settle for 12 h at 37°C. Reporter plasmids (100 ng; EMD Millipore, Billerica, MA, USA) containing wild-type (CCTTTGATC; TOP flash) or mutated (CCTTTGGCC; FOP flash) T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) binding sites used to determine β-catenin transcriptional activity (18) plus 1 ng pRL-TK Renilla plasmid were co-transfected using Lipofectamine 2000 according to the manufacturer’s protocol. Firefly and Renilla luciferase activity were measured 48 h following transfection using the Dual Luciferase Reporter Assay kit (Promega Corporation, Madison, WI, USA) according to manufacturer’s protocol. Luciferase activity was normalized to Renilla luciferase activity. All experiments were performed in triplicate.

Statistical analysis. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The one-way analysis of variance was performed, followed by Student-Newman-Keuls post-hoc test. The association between LASP2 expression and the clinicopathological characteristics was analyzed via Pearson’s χ^2 test. The overall survival was calculated as the time from the date of primary surgery to the date of death or the date of the last follow-up. Recurrent-free survival was defined as the time from the date of primary surgery to the date of first recurrence or the date of the last follow-up. Kaplan-Meier analysis and log-rank test were used to perform survival analyses. The clinicopathological characteristics was evaluated by two pathologists, and the association between variables (sex, age, tumor grade, tumor size, T classification using the TNM staging system and LASP2 expression level) survival rates were analyzed by the Cox proportional hazards model for univariate and multivariate analyses. P<0.05 indicated that the difference between groups was statistically significant.

Results

LASP2 is downregulated in patients with bladder cancer. To reveal the function of LASP2 in bladder cancer, RT-qPCR was performed to examine the LASP2 expression in bladder cancer tissues and cell lines. As presented in Fig. 1A, LASP2 mRNA expression was decreased in 75.0% (45/60) of patients. Furthermore, LASP2 was significantly decreased in bladder cancer cell lines compared with normal urothelial cells (Fig. 1B).

LASP2 is associated with the clinicopathological characteristics of patients with bladder cancer. The association between LASP2 expression and clinicopathological features was further investigated in bladder cancer patients. Statistical analyses...
revealed that the expression of LASP2 was significantly associated with tumor size and tumor stage (Table II). In the Kaplan-Meier analysis, the cut-off point (relative value, -2.34) of LASP2 mRNA expression was defined as the median (low LASP2 group, 49 months; high LASP2 group, 48 months). The results revealed that the overall and recurrent-free survival times of patients with low LASP2 expression was significantly shorter compared with those with high LASP2 expression (Fig. 1C). Univariate and multivariate analyses revealed that LASP2 expression was an independent prognostic factor for overall and recurrent-free survival in patients with bladder cancer (Tables III and IV).

LASP2 inhibits bladder cancer cell proliferation, migration and invasion, and angiogenesis in vitro. The effects of LASP2 on cell proliferation, migration and invasion, and angiogenesis were then evaluated in EJ and T24 cells through overexpressing and inhibiting LASP2 expression (Figs. 2 and 3). LASP2 overexpression was confirmed via western blotting (Fig. 2A). The EdU labeling assay revealed that upregulated LASP2 expression inhibited the proliferation of the two cell lines (Fig. 2B). In addition, the overexpression of LASP2 significantly decreased the ability of bladder cancer cells to induce tube formation by HUVECs (Fig. 2C). The results of wound healing and Transwell migration and Matrigel invasion assays suggested that tumor migration decreased following the transfection of LASP2 plasmids into EJ cells (Fig. 2D and E). Also, the Matrigel invasion assay suggested that tumor invasion decreased following the transfection of LASP2 plasmids into EJ cells (Fig. 2E). In contrast, LASP2 inhibition significantly promoted cell proliferation, invasion and migration and angiogenesis (Fig. 3).

LASP2 mediates the Wnt/β-catenin signaling pathway in bladder cancer cells. The molecular mechanism by which LASP2 exerts its biological function was further explored. As
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LASP2 upregulation significantly inhibited transactivation activity of β-catenin/TCF in the two cell lines using the luciferase reporter assay. Furthermore, the expression

Table IV. Multivariate analysis in patients with bladder cancer.

| Prognostic variables | Overall survival Hazard ratio (95% CI) P-value | Recurrent-free survival Hazard ratio (95% CI) P-value |
|----------------------|-----------------------------------------------|-----------------------------------------------------|
| Sex (male vs. female) | -                                             | -                                                   |
| Age (>60 vs. ≤60 years) | -                                             | -                                                   |
| Tumor grade (high vs. low) | 2.861 (1.576-5.196) 0.001 | -                                                   |
| Tumor size (≥3 vs. <3 cm) | -                                             | -                                                   |
| T classification (T2-4 vs. T1 and Ta) | 1.924 (1.024-3.614) 0.042 | -                                                   |
| LASP2 (high vs. low) | 0.298 (0.154-0.579) <0.001 | 0.585 (0.394-0.870) 0.008 |

CI, confidence interval; LASP2, LIM and SH3 Protein 2.

Figure 3. LASP2 downregulation promotes bladder cancer cell aggressiveness. (A) Western blotting analysis of LASP2 expression in LASP2 knockdown EJ and T24 cells. (B) Representative images (left panel) and quantification (right panel) of EdU incorporation in EJ and T24 bladder cancer cells. DAPI was used as a DNA stain. Representative images (left panel) and quantification (right panel) of (C) HUVECs cultured on Matrigel-coated plates with conditioned medium from LASP2-silenced cells, (D) migratory cells assessed using a wound healing assay and (E) migratory cells assessed using transwell migration and Matrigel invasion assays, respectively. *P<0.05. EdU, 5-ethynyl-2'-deoxyuridine; HUVEC, human umbilical vein endothelial cell; LASP2, LIM and SH3 Protein 2; Ri, RNA interference.

presented in Fig. 4A, LASP2 upregulation significantly inhibited, whereas LASP2 knockdown significantly increased the
levels of downstream targets of the Wnt/β-catenin signaling pathway, including c-Myc, c-Jun, cyclin D1 (CCND1), cytotoxic T lymphocyte-associated protein 4, lymphoid enhancer binding factor 1, T cell factor 1 and AXIN2 were markedly lower in LASP2-overexpressing tumor cells compared with that in LASP2-silenced cells (Fig. 4B). Western blotting assays were also performed to demonstrate that LASP2 expression was negatively associated with CCND1 expression and nuclear β-catenin expression (Fig. 5).

Discussion

The results of the current study demonstrated that LASP2 functions as a tumor suppressor in bladder cancer. Low LASP2 expression was frequently detected in bladder cancer, and was associated with larger tumor size, advanced TNM staging (i.e. T classification) and poor prognosis. Additionally, the overexpression of LASP2 attenuates the proliferation, migration and invasion of bladder cancer cells, and angiogenesis via the regulation of Wnt/β-catenin signaling pathway. These findings suggest that LASP2 may serve as a prognostic marker for patients with bladder cancer and an important role in bladder cancer progression.

Nebulin family members, with molecular weights ranging from 34-900 kDa, have diverse expression patterns and cellular functions (19). The majority of nebulin family members contain the defining characteristic of actin-binding domains referred to as ‘nebulin repeats’ through which the members form multi-domain proteins to interact with filamentous actin (20). The nebulin protein family has been proposed to serve essential physiological roles in human diseases. For example, mutations in nebulin were demonstrated to be the main cause of the human muscle disorder nemaline myopathy, and subsequent distal and core-rod myopathies (21). The abnormal expression of nebulette or N-RAP genes have been associated with dilated cardiomyopathy (22). As a shorter splice variant of nebulette, LASP2 is a novel nebulin family member (23). In cardiomyocytes, LASP2 is localized in different cytoskeletal structures, including the intercalated discs, Z-discs and focal adhesions, implying that LASP2 may act as a molecular scaffold that is important for cell migration and adhesion, and focal adhesion turnover (24,25).

Recent data revealed that LASP2 is associated with the development of human cancers. Wang et al. (14) recently demonstrated that LASP2 functions as a tumor suppressor gene in colon cancer and inhibits cancer progression via the c-Jun N-terminal kinase/p38 mitogen-activated protein kinase signaling pathway. In contrast, Zhang et al. (13) reported that LASP2 acts as an oncogene in non-small-cell lung carcinoma (NSCLC) and accelerates tumor invasion through facilitating the phosphorylation of focal adhesion kinase. These studies indicate the diverse roles of LASP2 in different cancers. In the present study, the downregulation of LASP2 expression in bladder cancer was detected. Additionally, LASP2 expression was associated with larger tumor size and advanced TNM staging. In addition, functional experiments indicated that LASP2 could attenuate bladder cancer aggression. Together, these findings indicate that LASP2 may perform different functions in different cancers.
Importantly, it was demonstrated that increased expression of LASP2 was associated with the reduction in the nuclear translocation of β-catenin in bladder cancer cells. Additionally, it was revealed that LASP2 downregulates the expression of downstream genes of the Wnt/β-catenin signaling pathway. Together, the findings revealed that the tumor-suppressing role of LASP2 in bladder cancer may be associated with Wnt/β-catenin signaling pathway inactivation. The findings highlighted the importance of the Wnt/β-catenin signaling pathway in bladder cancer progression, and may partially explain the different roles for LASP2 in different cancer types. Notably, it was demonstrated that the upregulation of LASP2 inhibited, whereas the knocking down of LASP2 induced HUVEC tube formation, revealing the role of LASP2 in angiogenesis.

The clinical significance of nebulin family proteins has been illustrated in previous studies. Qiu et al (26) demonstrated that nebulin overexpression in patients with colorectal cancer was associated with increased overall survival. Yang et al (27) suggested that LASP1 may serve as a prognostic biomarker for patients with clear cell renal cell carcinoma. Zhang et al (13) reported that the expression of LASP-2 was significantly correlated with poor prognosis in patients with NSCLC. The current study revealed that high LASP2 expression was associated with favorable overall and recurrent-free survival rates in patients with bladder cancer, further suggesting that LASP2 may be a marker used to predict the prognosis of patients with cancer.

The present study has several limitations. First, the retrospective, observational nature of the current study with unknown factors may affect the studied outcomes and were not captured in the present data collection. Second, only 196 patients were included; further studies on larger populations are required to confirm the preliminary results of the current study.

In summary, the present study has demonstrated that LASP2 has an important role in bladder carcinogenesis. LASP2 was revealed to mediate bladder cancer cell proliferation and metastasis, and angiogenesis in bladder cancer via the Wnt/β-catenin signaling pathway. In addition, LASP2 may function as an independent prognostic marker for bladder cancer. Thus, LASP2 could be a promising therapeutic target for the future treatment of bladder cancer.

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Availability of data and materials

All data analyzed during the present study are included in this manuscript.

Authors' contributions

RY participated in data collection and drafted the manuscript. ZL performed the statistical analysis. YC and JK participated in data collection and drafted the manuscript. YC and JK participated in data collection and drafted the manuscript. All data analyzed during the present study are included in this manuscript. The First People's Hospital of Jingmen (grant no. 20154567). The present study was supported by the Innovation Fund of the First People's Hospital of Jingmen (Jingmen, China). All patients provided written informed consent.

Ethics approval and consent to participate

The present study was approved by the Institutional Research Ethics Committee of the The First People's Hospital of Jingmen (Jingmen, China). All patients provided written informed consent.

Patient consent for publication

Written informed consent was obtained from each patient for the use of the tissue samples for paper publication.

Competing interests

The authors declare that they have no competing interests.

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