

Original Article

LRRC3B inhibits the proliferation and invasion of bladder cancer cells

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Abstract: Leucine-rich repeat (LRR)-containing 3B (LRRC3B) is a putative LRR-containing transmembrane protein and was down-regulated in many types of tumors. However, its expression and role in bladder cancer (BC) remains unclear. In our research, we aimed to assess the expression and function of LRRC3B in BC. Our results showed that the expression of LRRC3B was significantly reduced in BC cell lines. The *in vitro* experiments demonstrated that overexpression of LRRC3B inhibited the proliferation, migration and invasion of BC cells. Furthermore, *in vivo* xenograft tumor assay showed that LRRC3B attenuated tumor growth. Finally, LRRC3B inhibited the expression of β -catenin, cyclin D1 and c-Myc in BC cells. Taken together, these data indicated that LRRC3B inhibited BC cell proliferation and invasion partly by interfering with Wnt/ β -catenin signaling. Therefore, these findings reveal that LRRC3B may be a potential therapeutic target for the treatment of BC.

Keywords: Leucine-rich repeat (LRR)-containing 3B (LRRC3B), bladder cancer, migration, epithelial-mesenchymal transition (EMT)

Introduction

Bladder cancer (BC) is the second most common cancer of the genitourinary system [25]. Approximately 386,000 patients worldwide are diagnosed with BC, and approximately 150,000 patients die from this disease each year [10]. Despite many advances in BC research, there is still a pressing need for new therapies for treating BC. The majority of patients succumb to tumor metastasis. Thus, exploring the molecular mechanisms that regulate BC metastasis is urgently required.

LRR-containing proteins, of which there are > 2,000, participate in many important processes, including plant and animal immunity, hormone-receptor interactions, cell adhesion, signal transduction, regulation of gene expression, and apoptosis [12, 19]. Leucine-rich repeat (LRR)-containing 3B (LRRC3B) is a putative LRR-containing transmembrane protein [15]. A number of microarray expression profiling studies on human cancers have shown that LRRC3B was down-regulated in gastric [2], breast [20], colon [1], testis [16], prostate [17], and brain cancers [21], suggesting LRRC3B

involvement in carcinogenesis. For example, one study confirmed that LRRC3B expression was downregulated in human non-small-cell lung cancer (NSCLC) tissues and that its upregulation significantly inhibited proliferation, colony formation ability, and invasion in NSCLC cells [13]. However, the role of LRRC3B in BC is still unclear, so the purpose of this study was to investigate the expression and role of LRRC3B in BC. Our data indicated that LRRC3B inhibited BC cell proliferation and invasion partly by interfering with Wnt/ β -catenin signaling.

Materials and methods

Cell culture

Human bladder cancer cell lines (T24, EJ and J82) and human bladder epithelial immortalized SV-HUC-1 cell line were obtained from the Chinese Science Institute (Shanghai, China). The SV-HUC-1 cells were cultured in F12K medium (Sigma-Aldrich, St Louis, MO, USA), the T24, EJ and J82 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). These media were supplemented with 10% fetal bovine serum (FBS; TBD, Tianjin, China) and 1% penicil-

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lin/streptomycin (Hyclone). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from BC cells using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). Real-time PCR was performed using the Exicycler Quantitative Thermal Block (BiONEER). The RT reaction product (100 ng) was amplified in a 15- μ L reaction volume with 2 \times SYBR Premix EX Taq (Takara). We used the Primer3 program 10 to design the LRRC3B exon 2 forward (5'-TTCCCTCTCCATGTGTCTCC-3') and reverse (5'-CCAGCATGTTTCATCCAACAC-3') primers. Samples were heated to 95°C for 1 min and then amplified for 45 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 10 min. β -actin was used as an internal control. Relative quantification of LRRC3B mRNA was analyzed by the comparative threshold cycle (CT) method.

Western blot

Proteins were extracted using RIPA lysis buffer containing phosphatase and protease inhibitors (Sigma, St. Louis, MO, USA). Cells lysates were harvested and proteins were detected as previously described [27]. LRRC3B, E-cadherin, N-cadherin, vimentin, β -catenin, cyclin D1, c-myc and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). All primary antibodies were used at a dilution of 1:1000 and the secondary antibodies were used at 1:5000. The bands density was quantified with ImageJ software (National Institutes of health, Bethesda, MD).

Construction of plasmids and transfection

The full-length LRRC3B cDNA was amplified and sub-cloned into pcDNA3.1 (Invitrogen), whereas the empty vector pcDNA3.1 was used as control. T24 cells were transfected with LRRC3B or vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Cell proliferation assay

After treatments as designed, cells were dissociated from the culture wells with 0.25% trypsin

and counted using Bio-Rad cell counter (Bio-Rad Laboratories, Hercules, CA, USA). Then, 2000 cells/well were plated into a 96-well flat bottomed plate, after culture for 1, 2, 3 and 4 days, MTT solution (5 mg/ml) was added to each well. Approximately four to six hours were required to produce formazan, which was then dissolved in DMSO. The absorbance was measured at 490 nm using an ELX Ultra microplate reader (Bio-Tek, VT, USA).

Cell migration and invasion assays

Cell migration assay was carried out using transwell culture system. A total of 5×10^4 cells were plated into the upper chamber of the transwell chamber. The lower chamber was filled with culture medium. 24 h later, the non-migrated cells on the upper side of the membrane were removed by cotton swabs. The migrating cells on the lower side of the insert filter were fixed with formaldehyde for 10 min and stained the cells with 1% crystal violet solution for 20 min. The number of cells was counted and imaged using the light microscope (Olympus, Tokyo, Japan). The invasion assay with performed essentially as detailed before with minor modifications. T24 cells were trypsinized and suspended in 0.5 ml of serum-free medium. The cell suspensions were seeded onto the membranes of the upper chambers of modified Boyden chambers (8 μ m; Corning Costar, Cambridge, MA, USA) which were pre-coated with Matrigel. The lower chambers contained the same concentrations of the used agents in 10% FBS-containing medium. The cells were then incubated at 37°C for 24 h. After incubation, the cells remaining on the upper surface of the membranes were removed gently with cotton swabs. Cells which invaded into the lower surface of the membrane were fixed in 90% methanol and stained with 0.1% crystal violet. Stained cells were counted in at least ten 200 \times fields.

In vivo xenograft tumor assay

Six-week-old male Balb/c nude mice (4-5 weeks of age, 18-20 g) were purchased from the Laboratory Animal of the Huaihe Hospital, Henan University (China) and maintained in plastic cages in a temperature-controlled room on a 12-h light/dark cycle with free access to water and a standard pellet diet throughout the experiment. After an acclimation period of 7 d,

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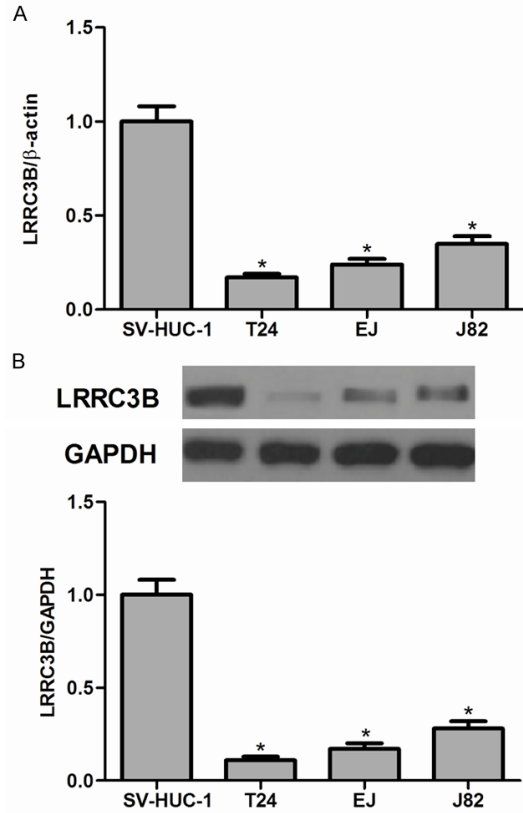


Figure 1. LRRC3B was frequently silenced in human BC cell lines. A. The mRNA level of LRRC3B in 3 kinds of cell lines (T24, EJ and J82) was significantly reduced than that in SV-HUC-1. * $P < 0.05$. B. The protein level of LRRC3B in 3 kinds of cell lines (T24, EJ and J82) was significantly decreased than that in control cells (SV-HUC-1). * $P < 0.05$.

the solid tumor was developed by the subcutaneous inoculation of 1×10^6 T24 cells on the right flank of each mouse. The tumor volumes were calculated in each group every one week. All the animals were sacrificed 21 d after tumor inoculation. All animal experiments were performed in a humane manner after approval from the Institutional Experiment Committee of the University of Huaihe Hospital, Henan University.

Statistical analysis

The data are expressed as the mean \pm standard deviation (SD) of three different tests. Statistical analysis was conducted by one-way ANOVA using the Prism statistical software package (GraphPad Software, USA). Statistical analysis was defined as significant if * $P < 0.05$.

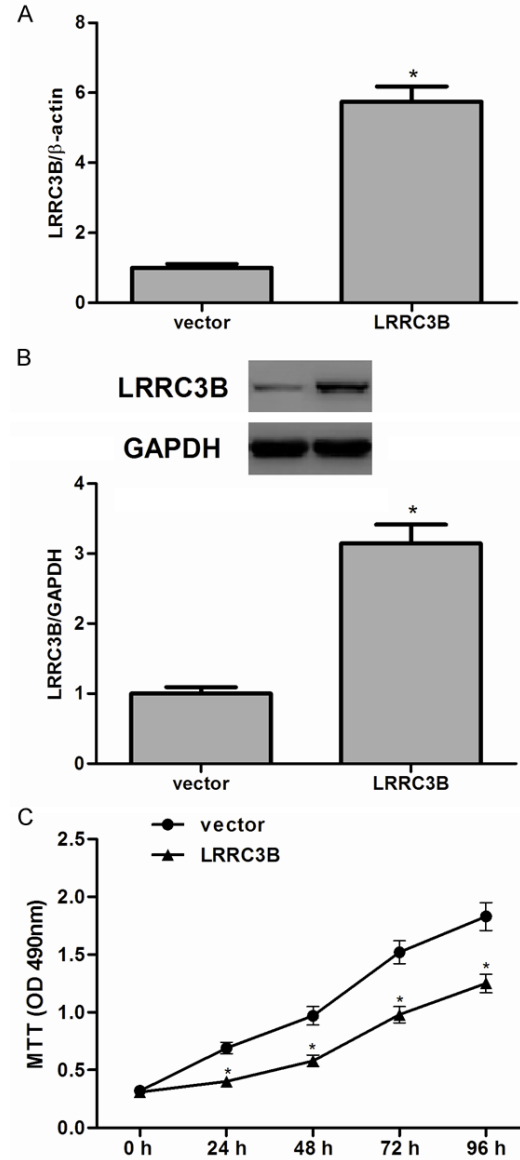


Figure 2. LRRC3B inhibited BC cell proliferation *in vitro*. T24 cells were transfected with LRRC3B or vector for 24 h, respectively. A. The mRNA expression of LRRC3B was detected using qRT-PCR. B. The protein expression of LRRC3B was evaluated using western blot. C. Cell proliferation was measured by the MTT assay. * $P < 0.05$.

Results

LRRC3B was frequently silenced in human BC cell lines

We examined LRRC3B expression LRRC3B expression in three BC cell lines (T24, EJ and J82) and normal human bladder epithelial immortalized SV-HUC-1 cell line by RT-PCR and western blot. As indicated in

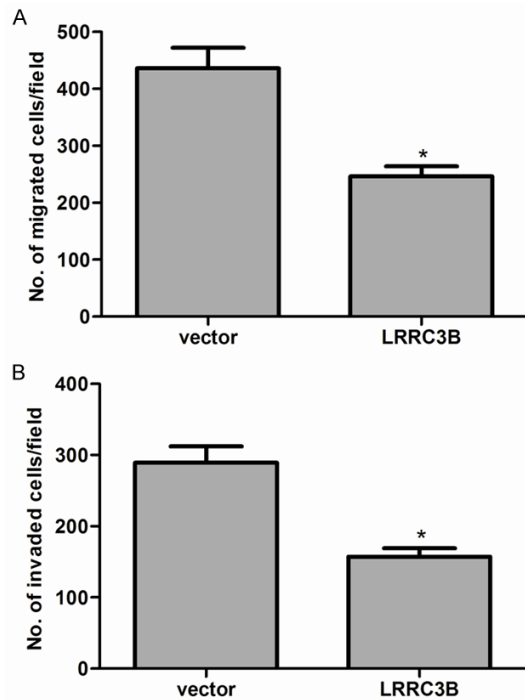


Figure 3. LRRC3B inhibited BC cell migration and invasion *in vitro*. T24 cells were transfected with LRRC3B or vector for 24 h, respectively. A. Cell migration was evaluated using the transwell migration assay. B. Cell invasion was determined using the transwell invasion assay. *P < 0.05.

Figure 1A, the expression of LRRC3B at the mRNA level was significantly reduced in the three BC cell lines in comparison with that in the normal cell line SV-HUC-1. Consistently, western blot analysis showed the similar results (P < 0.05, **Figure 1B**).

LRRC3B inhibited BC cell proliferation *in vitro*

To examine the possible activity of LRRC3B as a tumor suppressor, we established stably transfected LRRC3B-expressing T24 cell line. As the following result, the mRNA expression of LRRC3B was greatly upregulated in transfected LRRC3B-expressing T24 cells (**Figure 2A**). Similarly, LRRC3B protein expression was higher than control group (**Figure 2B**). The effect of the LRRC3B on the proliferation of T24 cells was detected by the MTT assay. The experimental data indicated that LRRC3B efficiently suppressed the proliferation of T24 cells (**Figure 2C**).

LRRC3B inhibited BC cell migration and invasion *in vitro*

Next, we examined the effects of LRRC3B on cell migration and invasion using the Transwell

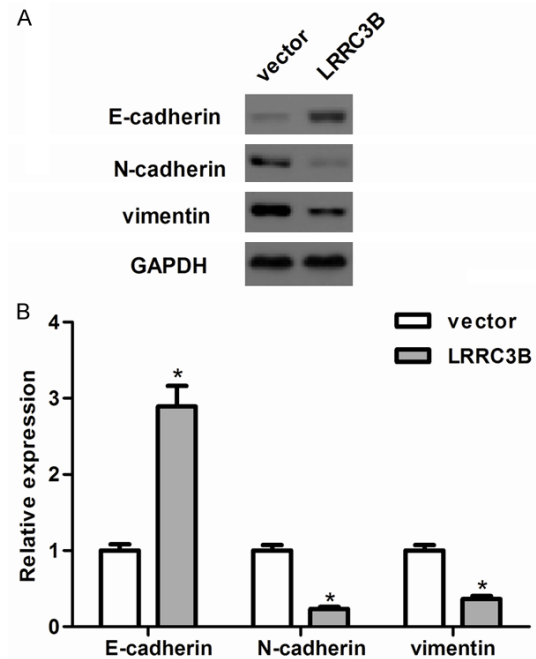


Figure 4. LRRC3B inhibits EMT phenotype in BC cells. T24 cells were transfected with LRRC3B or vector for 24 h, respectively. Western blot analysis was used to evaluate the protein levels of E-cadherin, N-cadherin and vimentin in T24 cells. Quantification analysis was performed using ImageJ software. *P < 0.05.

migration and invasion system. The transwell migration assay showed that the number of migrated cells was sharply lower in the up-regulated of LRRC3B group than in the control group (**Figure 3A**). In addition, the results of invasion assay indicated that up-regulated of LRRC3B obviously suppressed the invasion of T24 cells (**Figure 3B**).

LRRC3B inhibited EMT phenotype in BC cells

Furthermore, we observed that compared to vector group, the E-cadherin protein level in up-regulated-LRRC3B cells was significantly increased. In contrast, the expression of N-cadherin and vimentin was significantly lower in LRRC3B-expressing T24 cells than that in vector-transfected cells (**Figure 4**).

LRRC3B inhibited the growth of BC *in vivo*

In order to examine the effect of LRRC3B on BC growth *in vivo*, we next examined the effect of LRRC3B overexpression by a tumorigenesis assay in nude mice. Nude mice were injected with the stably transfected LRRC3B-expressing T24 cells or empty vector-transfected cells. The control cells formed rapidly growing tumors,

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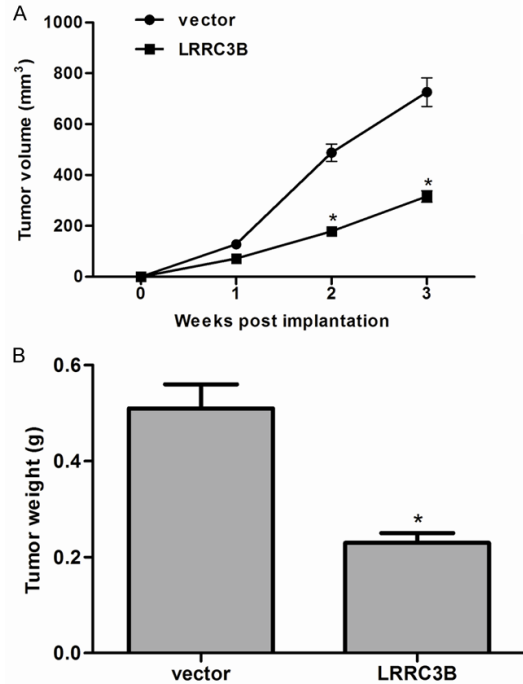


Figure 5. LRRC3B inhibited the growth of BC *in vivo*. Nude mice were injected with the stably transfected LRRC3B-expressing T24 cells or empty vector-transfected cells. A. The tumor volumes were calculated in each group every one week. B. After 3 weeks after injection, the tumors were dissected and weighed. *P < 0.05.

whereas the LRRC3B cells formed tumors that were much smaller (Figure 5A). In addition, we observed that LRRC3B significantly reduced the weight of tumors (Figure 5B).

LRRC3B inhibited the Wnt/ β -catenin pathway in BC cells

It has been shown that the dysregulation of Wnt signaling contributes to the development of human cancers, including BC. Therefore, we tested the effect of LRRC3B on the expression of β -catenin, cyclin D1 and c-myc in T24 cells. As shown in Figure 6, the protein expression levels of β -catenin, cyclin D1 and c-myc were significantly down-regulated in LRRC3B-transfected T24 cells, compared with the vector group.

Discussion

In this study, we showed that LRRC3B was involved in the pathogenesis of BC. LRRC3B was down-regulated in BC cell lines; Overexpression of LRRC3B inhibited BC cell

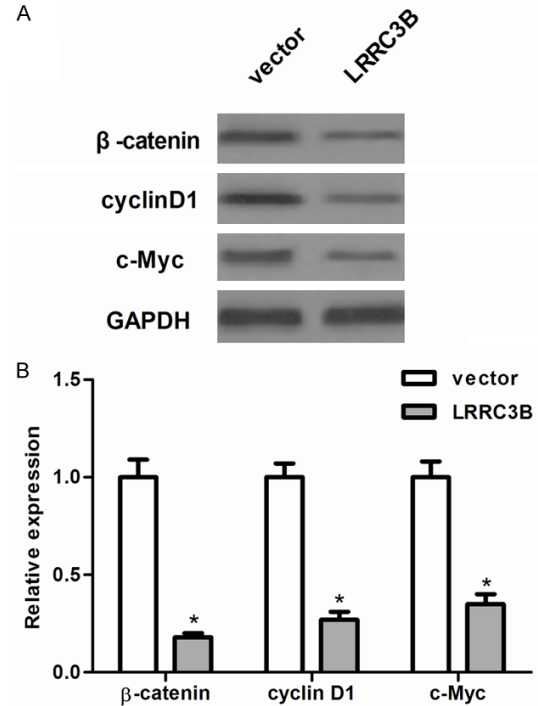


Figure 6. LRRC3B inhibited the Wnt/ β -catenin pathway in BC cells. T24 cells were transfected with LRRC3B or vector for 24 h, respectively. Western blot analysis was used to evaluate the protein levels of β -catenin, cyclin D1 and c-myc in T24 cells. Quantification analysis was performed using ImageJ software. *P < 0.05.

proliferation and invasion, as well as reduced the growth of BC *in vivo*. In addition, up-regulation of LRRC3B inhibited EMT phenotype in BC cells. Finally, LRRC3B inhibited the Wnt/ β -catenin pathway in BC cells.

LRRC3B is a potential tumor suppressor gene newly discovered; however, the specific biologic function is still unknown. The abnormal expression of LRRC3B could serve as a useful marker for diagnosis and prognosis in breast carcinomas [24]. In colorectal cancer, the expression of LRRC3B was significantly lower in colorectal cancer tissues than in the corresponding non-cancerous tissues [5, 22]. In addition, the study from Haraldson K showed that LRRC3B strongly suppressed cell growth *in vitro* in KRC/Y renal cell carcinoma line [11]. These findings are consistent with our research showing that LRRC3B was down-regulated in BC cell lines; and overexpression of LRRC3B inhibited BC cell proliferation *in vitro*, as well as reduced the growth of BC *in vivo*. All these data suggest that LRRC3B may act as a tumor suppressor in the progression of BC.

The expression and function of LRRC3B in bladder cancer

Local metastasis is one of the primary reasons for clinical treatment failure in malignant BC [6, 14]. Several studies showed that LRRC3B was closely related with tumor metastasis and invasion. For example, stable transfection of LRRC3B significantly inhibited the invasion in NSCLC cells [13]. Similarly, herein, we observed that overexpression of LRRC3B inhibited BC cell migration and invasion. EMT is a critical step in the acquisition of the migratory and invasive capabilities. It is characterized by repression of epithelial markers and induction of mesenchymal markers, and reduction or a loss of E-cadherin expression is one of the well-established hallmarks of EMT [18, 26]. Our research indicated that LRRC3B inhibited EMT phenotype in BC cells as a tumor suppressed factor. These data imply that LRRC3B negatively regulates the EMT phenotype, consequently markedly affects BC cell migration and invasion *in vitro*.

Wnt/ β -catenin signal transduction pathway is constitutively activated in several major human cancers, including BC [7, 23]. Growing body of evidence suggests that the activation of Wnt/ β -catenin pathway plays an important role for metastasis and development of BC [4, 8, 9]. It was reported that knockdown of protein disulfide isomerases A6 markedly suppressed BC proliferation and invasion through decreasing the protein expression of β -catenin, cyclin D1 and c-Myc, and thus suppressed the Wnt/ β -catenin signaling pathway [3]. Consistent with the previous studies, the results in the present study showed that overexpression of LRRC3B down-regulated the protein expression levels of β -catenin, cyclin D1 and c-myc in T24 cells. These data imply that LRRC3B inhibited BC cell proliferation and invasion, at least in part, through suppressing the Wnt/ β -catenin signaling pathway.

In conclusion, our findings showed that LRRC3B can efficiently inhibit BC cell proliferation and invasion, at least in part, through suppressing the Wnt/ β -catenin signaling pathway. Therefore, LRRC3B may be a good molecular target for the prevention and treatment of BC.

Disclosure of conflict of interest

None.

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References

- [1] Bianchini M, Levy E, Zucchini C, Pinski V, Macagno C, De Sanctis P, Valvassori L, Carinci P, Mordoh J. Comparative study of gene expression by cDNA microarray in human colorectal cancer tissues and normal mucosa. *Int J Oncol* 2006; 29: 83-94.
- [2] Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, Chan AS, Law S, Troyanskaya OG, Wong J, So S, Botstein D, Brown PO. Variation in gene expression patterns in human gastric cancers. *Mol Biol Cell* 2003; 14: 3208-3215.
- [3] Cheng HP, Liu Q, Li Y, Li XD, Zhu CY. The inhibitory effect of PDIA6 downregulation on bladder cancer cell proliferation and invasion. *Oncol Res* 2017; 25: 587-593.
- [4] Costa VL, Henrique R, Ribeiro FR, Carvalho JR, Oliveira J, Lobo F, Teixeira MR, Jerónimo C. Epigenetic regulation of Wnt signaling pathway in urological cancer. *Epigenetics* 2010; 5: 343-351.
- [5] Demokan S, Chuang AY, Pattani KM, Sidransky D, Koch W, Califano JA. Validation of nucleolar protein 4 as a novel methylated tumor suppressor gene in head and neck cancer. *Oncol Rep* 2014; 31: 1014-1020.
- [6] Dmitriev AA, Kashuba VI, Haraldson K, Senchenko VN, Pavlova TV, Kudryavtseva AV, Anedchenko EA, Krasnov GS, Pronina IV, Loginov VI, Kondratieva TT, Kazubskaya TP, Braga EA, Yenamandra SP, Ignatjev I, Ernberg I, Klein G, Lerman MI, Zbarovsky ER. Genetic and epigenetic analysis of non-small cell lung cancer with NotI-microarrays. *Epigenetics* 2012; 7: 502-513.
- [7] Du HF, Ou LP, Lv CK, Yang X, Song XD, Fan YR, Wu XH, Luo CL. Expression of hepaCAM inhibits bladder cancer cell proliferation via a Wnt/ β -catenin-dependent pathway *in vitro* and *in vivo*. *Cancer Biol Ther* 2015; 16: 1502-1513.
- [8] Urakami S, Shiina H, Enokida H, Kawakami T, Tokizane T, Ogishima T, Tanaka Y, Li LC, Ribeiro-Filho LA, Terashima M, Kikuno N, Adachi H, Yoneda T, Kishi H, Shigeno K, Konety BR, Igawa M, Dahiya R. Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/ β -catenin signaling pathway. *Clin Cancer Res* 2006; 12: 383-391.
- [9] Du Y, Wang Y, Zhang F, Wu W, Wang W, Li H, Xia S, Liu H. Regulation of metastasis of bladder cancer cells through the WNT signaling pathway. *Tumor Biol* 2015; 36: 8839-8844.

The expression and function of LRRC3B in bladder cancer

- [10] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-2917.
- [11] Haraldson K, Kashuba VI, Dmitriev AA, Senchenko VN, Kudryavtseva AV, Pavlova TV, Braga EA, Pronina IV, Kondratov AG, Rynditch AV, Lerman MI, Zabarovsky ER. LRRC3B gene is frequently epigenetically inactivated in several epithelial malignancies and inhibits cell growth and replication. *Biochimie* 2012; 94: 1151-1157.
- [12] Kajava AV. Structural diversity of leucine-rich repeat proteins. *J Mol Biol* 1998; 277: 519-527.
- [13] Kan L, Zhang M, He P. LRRC3B is downregulated in non-small cell lung cancer and connected with cell proliferation and invasion. *Zhongguo Fei Ai Za Zhi* 2016; 19: 177-183.
- [14] Nakagawa T, Kanai Y, Nakanishi H, Komiyama M, Fujimoto H. Characteristics of lymph node metastases defining the outcome after radical cystectomy of urothelial bladder carcinoma. *Jpn J Clin Oncol* 2012; 42: 1066-1072.
- [15] Kim M, Kim JH, Jang HR, Kim HM, Lee CW, Noh SM, Song KS, Cho JS, Jeong HY, Hahn Y, Yeom YI, Yoo HS, Kim YS. LRRC3B, encoding a leucine-rich repeat-containing protein, is a putative tumor suppressor gene in gastric cancer. *Cancer Res* 2008; 68: 7147-7155.
- [16] Korkola JE, Houldsworth J, Chadalavada RS, Olshen AB, Dobrzynski D, Reuter VE, Bosl GJ, Chaganti RS. Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. *Cancer Res* 2006; 66: 820-827.
- [17] Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevad L, Rayford W, Bergerheim U, Ekman P, DeMarzo AM, Tibshirani R, Botstein D, Brown PO, Brooks JD, Pollack JR. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *P Natl Acad Sci U S A* 2004; 101: 811-816.
- [18] McConkey DJ, Choi W, Marquis L, Martin F, Williams MB, Shah J, Svatek R, Das A, Adam L, Kamat A, Siefker-Radtke A, Dinney C. Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. *Cancer Metast Rev* 2009; 28: 335-344.
- [19] Pancer Z, Cooper MD. The evolution of adaptive immunity. *Ann Rev Immunol* 2006; 24: 497-518.
- [20] Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* 2006; 9: 121-132.
- [21] Sun L, Hui AM, Su Q, Vortmeyer A, Kotliarov Y, Pastorino S, Passaniti A, Menon J, Walling J, Bailey R, Rosenblum M, Mikkelsen T, Fine HA. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell* 2006; 9: 287-300.
- [22] Tian XQ, Zhang Y, Sun D, Zhao S, Xiong H, Fang J. Epigenetic silencing of LRRC3B in colorectal cancer. *Scand J Gastroentero* 2009; 44: 79-84.
- [23] Urakami S, Shiina H, Enokida H, Kawakami T, Tokizane T, Ogishima T, Tanaka Y, Li LC, Ribeiro-Filho LA, Terashima M, Kikuno N, Adachi H, Yoneda T, Kishi H, Shigeno K, Konety BR, Igawa M, Dahiya R. Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/beta-catenin signaling pathway. *Clin Cancer Res* 2006; 12: 383-391.
- [24] Wang Y, Peng Y, Zhou Y, Zhou Y, Zhong H. The clinical value of LRRC3B gene expression and promoter hypermethylation in breast carcinomas. *Cell Biochem Bioph* 2014; 70: 1035-1041.
- [25] Wu Z, Wang C, Zhang Z, Liu W, Xu H, Wang H, Wang Y, Zhang W, Wang SL. High expression of Derlin-1 is associated with the malignancy of bladder cancer in a Chinese Han population. *PLoS One* 2016; 11: e0168351.
- [26] Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metast Rev* 2009; 28: 15-33.
- [27] Zhang XJ, Yu HY, Cai YJ, Ke M. Lycium barbarum polysaccharides inhibit proliferation and migration of bladder cancer cell lines BIU87 by suppressing Pi3K/AKT pathway. *Oncotarget* 2017; 8: 5936-5942.