# B7-H3 promotes malignant progression of muscle-invasive bladder cancer

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**Abstract.** The objective of the present study was to investigate the expression of B7 homologue 3 (B7-H3) in muscle-invasive bladder cancer (MIBC) tissues, evaluate its correlation with patient clinicopathological characteristics, and to explore the effect of B7-H3 on MIBC cells. B7-H3 expression levels in tumor tissues from 115 patients undergoing radical cystectomy for MIBC were detected by immunohistochemical staining, followed by analysis of the association with clinicopathological characteristics and survival. A B7-H3-silenced cell line was established by RNA interference (RNAi). Alterations in cell proliferation, cell cycle, migration and invasion were analyzed *in vitro*. The proteins associated with cancer cell behavior were detected by western blot analysis. In addition, we utilized a xenograft tumor assay in nude mice to test the inhibitory effect of B7-H3 shRNA on MIBC *in vivo*. The results revealed that, among the 115 patients, the B7-H3 expression level was significantly associated with an increased incidence of distant metastasis (P=0.014) and vascular invasion (P=0.031), whereas it was not statistically associated with sex, age, pathologic grade, tumor stage, recurrence and lymphatic metastasis. Overall survival (OS) and progression-free survival (PFS) were significantly worse for patients with high B7-H3 expression (P<0.001 and P<0.001, respectively) among the 115 MIBC patients. Suppression of B7-H3 significantly inhibited the proliferation, caused G2 phase arrest, as well as declined migration and invasion abilities *in vitro*. The protein expression of Ki67, proliferating cell nuclear antigen (PCNA), matrix metalloproteinase 2 (MMP2) and MMP9 were decreased in the T24/B7-H3 shRNA group compared with the control (P<0.05, respectively). Finally, we were able to inhibit tumor development by decreasing B7-H3 expression *in vivo*. In conclusion, a high expression level of B7-H3 in MIBC tissues is associated with a poor clinicopathological status and poor prognosis, and promotes the development of MIBC *in vitro* and *in vivo*. Thus, B7-H3 may be a potential novel biomarker for the poor prognosis of MIBC patients.

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*Key words:* B7-H3, clinicopathological characteristics, muscle-invasive bladder cancer, RNA interference, *in vitro*, *in vivo*

## Introduction

Bladder cancer is a malignancy with the highest incidence in the urinary system; 79,030 Americans were diagnosed with bladder cancer in 2017, and 16,870 of them will succumb to the disease (1). Urothelial carcinoma is the most common pathologic type of bladder cancer, and muscle-invasive bladder cancer (MIBC) is a commonly occurring disease with a high mortality rate despite comprehensive treatment (2-7). Patients with MIBC are at risk for local invasion and distant metastasis, and are usually diagnosed at advanced stages and have a poor prognosis (2-7). With the increasing number of patients and the growing complexity of MIBC, biomarkers are required urgently to predict tumor progression, yet the development of practical and useful biomarkers for prognostication has been stagnated even further (4-7).

B7-H3 (B7 homologue 3), which has been recently discovered as a novel member of the B7 family molecules, plays an immuno-regulatory role between tumor and immune cells (8,9). B7-H3 is widely expressed in urologic tumors as well as in other human malignancies, and it is closely related to tumor progression, metastasis, recurrence and other adverse clinical features (10-19). Currently, the impact of B7-H3 on cancer progression has received increasing attention, although its co-stimulatory or co-inhibitory effect remains contentious (16-28). However, the role of B7-H3 on tumor progression in MIBC is not clear.

In the present study, we focused on the association between the expression level of B7-H3 and the malignant progression and poor survival in MIBC patients with 10 years follow up, and we analyzed their associations with various clinicopathological characteristics. Survival analysis was performed to determine the prognostic significance of B7-H3 expression on postoperative survival of patients and to evaluate potential effects on the progression of MIBC. Moreover, our study showed that suppression of B7-H3 inhibited proliferation, migration and invasion *in vitro* and tumor growth *in vivo*, and provided some significant findings for targeted therapy.

## Materials and methods

*Specimen collection*. Following Institutional Review Board's approval, the retrospective study enrolled 115 MIBC patients undergoing standard radical cystectomy in the Department of Urology, Fourth Hospital of Hebei Medical University (Hebei, China), between 2005 and 2006. Patients were classified according to the 2009 UICC TNM staging as well as in compliance with 2004 WHO/ISUP classification (2-5). All patients had no history of preoperative radiotherapy and/or chemotherapy, nor neoadjuvant chemotherapy. The participants provided their written informed consents. All patients did not manifest signs of tumor metastasis, as evidenced by cross-sectional imaging.

*Ethics statement*. Human samples and animals in this study were approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. The research involving human participants was approved by the Fourth Hospital of Hebei Medical University and the equivalent committee. The participants provided written informed consent before enrollment in this study. The consent forms were not be provided in this article due to the large number and they were all written in the Chinese language. The animal study was conducted with the approval of the Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University.

*Immunohistochemistry and scoring*. Specimens were fixed using formalin, followed by paraffin embedding and were sliced into 5-$\mu$m sections. The latter were dewaxed by xylene and dehydrated with gradient ethanol, heated in 1 mmol/l EDTA (pH 8.0) to 121˚C, cooled to 90˚C, and incubated for 5 min. Then, the sections were immerged into 3% $H_2O_2$ deionized water for 10 min to eliminate endogenous peroxidase activity and sealed with goat serum (cat. no. ZLI-9021; ZSGB-BIO, Beijing, China) for 30 min. After that, the immunohistochemistry for B7-H3 was carried out on consecutive sections according to standard pathologic procedures, with the primary mouse anti-human B7-H3 antibody (1:500 dilution; cat. no. ab105922; Abcam, Cambridge, UK) and goat anti-mouse IgG (cat. no. ZB-2305; ZSGB-BIO), then it was dripped with diaminobenzidine (DAB) for rendering for 3-5 min. Next, sections were then counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and coverslipped. PBS was used to replace the primary antibody as the negative control.

The scoring method of B7-H3 expression was based on the stained area and intensity of staining (20). Quantification was made as follows: Negative/weak, moderate, and strong intensity of B7-H3 expression. Sections with negative/weak intensity were classified as showing low B7-H3 expression, whereas sections with moderate and strong intensity were categorized as high B7-H3 expression (20).

*Cell lines and cell culture*. The human bladder cancer T24 and 5637 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured according to the instructions from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco; 4 weeksc, Inc., Waltham, MA, USA) and incubated at 37˚C in 5% $CO_2$.

*Generation of stable cell line*. The human B7-H3 (gene ID: 80381) targeting small hairpin (sh) RNA sequence 5'-TCGTGT GCTGGAGAAAGATCAAACAGAGC-3' (LV-B7-H3-RNAi) and an OnTargeted and control sequence 5'-GCACTACCA GAGCTAACTCAGATAGTACT-3' (control shRNA) were used to generate recombinant lentivirus (10) (purchased from GeneChem Co., Ltd., Shanghai, China). As the carrier, recombinant lentivirus GV248 was titered to $1x10^8$ TU/ml, and the multiplicity of infection (MOI) was 20. The lentivirus vector was able to express enhanced green fluorescent protein (EGFP). Reverse transcription-polymerase chain reaction (RT-PCR), western blotting, viability and invasion assays were performed to confirm the effect of B7-H3 silencing.

*Gene expression of B7-H3, RT-PCR, and western blot analysis*. Total RNA was extracted and reverse transcribed to produce cDNA. Forward and reverse primers were as follows: 5'-CCC ACAGGTTGCTTTGCTTAA-3' and 5'-GCAGACCCCTGG AGAACCA-3' (B7-H3); 5'-CAGCTCACCATGGATGAT GATATC-3' and 5'-AGCCGGCCTTGCACAT-3' (β-actin). The PCR conditions were 94˚C for 2 min, then 36 cycles or 28 cycles (B7-H3: 36 cycles; β-actin: 28 cycles) at 94˚C for 30 sec, 56˚C or 59˚C (B7-H3: 56˚C; β-actin: 59˚C) for 30 sec, 72˚C for 30 sec, and finally 72˚C for 2 min. The nuclear and cytoplasmic protein was extracted and concentrated and then determined using the bicinchoninic acid (BCA) method. A total of 50 $\mu$g of protein was transferred onto nitrocellulose filter membranes after 10% SDS-PAGE. The membranes were blocked and incubated with primary antibodies, including rabbit anti-B7-H3 (1:200 dilution; cat. no. ab134161; Abcam, Cambridge, UK), mouse anti-β-actin (1:1,000 dilution; cat. no. ab8226; Abcam), rabbit anti-Ki67 (1:1,000 dilution; cat. no. ab16667; Abcam), mouse anti-proliferating cell nuclear antigen (PCNA) (1:500 dilution; cat. no. ab29; Abcam), mouse anti-MMP2 (1:1,000 dilution; cat. no. ab37150; Abcam), and mouse anti-MMP9 (1:1,000 dilution; cat. no. ab38898; Abcam) monoclonal antibodies overnight at 4˚C, and then incubated with the secondary antibody (polyclonal goat anti-rabbit cat. no. 605-457-013/mouse cat. no. 605-301-002; 1:10,000 dilution) (Rockland Immunochemicals Inc., Limerick, PA, USA) for 1 h. Finally, the gray values were analyzed using Odyssey V3.0 software (http://www.filedudes.com/Odyssey_ Browser-download-175211.html).

*Cell proliferation*. Colony formation assay was used to test the ability of proliferation. For this assay, 6-well plates were used

to seed cell suspensions (300 cells/well). After incubation, cells were fixed in methyl hydrate for 10 min. Then the colonies were stained with Crystal violet and counted using an optical microscope (ContourGT-K 3D Optical Microscope; Brook Technology Co., Ltd., Beijing, China).

*3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay*. MTT assay was also used to observe and compare cell proliferative ability. A total of $2x10^3$ cells were plated into a well of 96-well plates, and 10 $\mu$l of 5 mg/ml MTT was added into each well and continued to culture for 4 h. Then after dimethyl sulfoxide addition, the plates were placed on a microplate autoreader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value.

*Cell cycle analysis*. Cells from the two different groups as previously described were at serum starvation for 24 h and then digested by trypsin followed by washing with PBS buffer. After that, the cells were fixed with 75% ethanol at 4˚C overnight. After the centrifugation was performed at 300 x g for 5 min, 1 ml 1X PBS was used for re-suspension. RNase A (0.5 ml) was then applied to the re-suspended cells for 20 min and subsequently stained with 1 mM PI at 37˚C for 15 min. Finally, cell cycle distribution of the different groups was detected by flow cytometry (Becton-Dickinson; BD Biosciences, San Jose, CA, USA) and cell cycle distribution was analyzed with Summit 4.3 software (Beckman Coulter, Inc., Brea, CA, USA).

*Cell scratch assays*. Cells were seeded to full confluency in 6-well plates. A scratch was introduced in the middle of each well using a sterile pipette tip. The medium was discarded and replaced with a fresh one. The rate of migration towards the center of the wound was determined at the indicated time points using vernier caliper (72 h).

*Cell invasion assays*. The invasion assays were performed with an 8.0-$\mu$m pore inserts in a 24-well Transwell chambers (Corning Costar Inc., Corning, NY, USA). For this assay, $2x10^5$ cells were isolated and added to the upper chamber of a Transwell coated with Matrigel (BD Bioscience, Mountain View, CA, USA). RPMI-1640 medium with 10% FBS was added to the lower chamber and incubated for 24 h. Cells that had migrated to the bottom of the filter were stained with a three-step stain set (Thermo Fisher Scientific, Inc., Manassas, VA, USA). The cells in each chamber were counted under a ContourGT-K 3D optical microscope (Brook Technology Co., Ltd.).

*Xenograft assays in vivo*. Sixteen female Athymic BALB/c nude mice (age, 5 weeks; weight, 20 g) were provided by Slac Laboratory Animal Co., Ltd. (Shanghai, China), and they were housed in a pathogen-free animal facility at 20˚C under an independent filtration airtight fume hood with 12-h light/12-h dark cycle with access to distilled food and water *ad libitum*, and randomly assigned to groups (8 mice/group). A total of $2x10^6$ cells were injected subcutaneously into nude mice and the tumor volume was then measured. Mice were divided into two groups which were treated differently using T24/control cells and T24/B7-H3 shRNA cells. The tumors were measured by Vernier caliper on day 14, 17, 21, 23, 26 and 29. Twenty-nine days after inoculation, the mice were sacrificed by cervical dislocation, and the final volume of tumor tissues was determined. The following formula was used for calculating tumor volume (V): V ($mm^3$) = tumor length (mm) x tumor width $(mm)^2/2$. To investigate the correlation between B7-H3 and tumor metastasis, T24/control cells and T24/B7-H3 shRNA cells were injected into the tail vein of mice, respectively. After 4 weeks, metastasis in the lung was detected and the tumors were harvested. The animal study was conducted with approval of the Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal surgery was performed with care to alleviate pain.

*Statistical analysis*. Data were analyzed with SPSS 22.0 software (IBM Corp., Armonk, NY). For the immunohistochemistry experiments, associations between B7-H3 expression level and the clinicopathological features were evaluated using $\chi^2$ tests. Associations of survival and tumor progression with B7-H3 expression were estimated by Kaplan-Meier method and log-rank tests. For *in vitro* and *in vivo* experiments using T24 cells, data are shown as the mean ± standard deviation (SD) of 3 repetitions. Student's t-test was used for statistical comparisons. A value of $P<0.05$ was assigned to indicate statistical significance.

## Results

*Associations between B7-H3 expression level and clinicopathological features in MIBC patients*. Weak, moderate and strong intensity of B7-H3 expression are shown in Fig. 1. In addition, high B7-H3-positive expression was found in 79/115 (68.7%) cases, and low B7-H3 expression was observed in 36/115 (31.3%) cases (Table I). High B7-H3 expression was significantly associated with distant metastasis (P=0.014) and vascular invasion (P=0.031) compared to the low-expression group. No significant associations were identified between B7-H3 expression and other pathological factors, including sex, age, tumor grade, tumor stage, recurrence and lymph node metastasis (all $P>0.05$, Table I).

*Associations between B7-H3 expression level and clinical outcomes in MIBC patients*. In our 10 year follow-up study, Kaplan-Meier survival analysis revealed that the estimated cancer overall survival (OS) rates (standard error) at 1, 5 and 10 years in patients with high B7-H3 expression were 98.7% (1.3%), 4.5% (4.9%) and 0% (0%) in contrast to 100% (0%), 58.1% (8.7%) and 48.7% (9.9%) in patients with low B7-H3 expression, respectively. The estimated cancer progression-free survival (PFS) rates at 1, 5 and 10 years following RC in patients with high B7-H3 expression were 71.8% (5.1%), 16.8% (4.6%), 0% (0%) in contrast to 94.4% (3.8%), 52.0% (9.2%), 43.4% (11.0%) in patients with low B7-H3 expression, respectively. Therefore, high B7-H3 expression was significantly associated with a much shorter OS and PFS in 115 MIBC patients ($P<0.001$, $P<0.001$; Table II and Fig. 1B and C).

Table I. Association of B7-H3 expression and clinicopathological characteristics of the patients with MIBC (N=115).

| Features | No. of patients | B7-H3 expression | | $\chi^2$ | P-value |
|---|---|---|---|---|---|
| | | Low n=36 | High n=79 | | |
| Age (years) | | | | 3.565 | 0.059 |
| <65 | 46 | 19 | 27 | | |
| ≥65 | 69 | 17 | 52 | | |
| Sex | | | | 0.344 | 0.558 |
| Male | 99 | 32 | 67 | | |
| Female | 16 | 4 | 12 | | |
| Tumor stage | | | | 3.789 | 0.052 |
| T2 | 31 | 14 | 17 | | |
| T3/T4 | 84 | 22 | 62 | | |
| Tumor grade | | | | 1.981 | 0.159 |
| Low | 23 | 10 | 13 | | |
| High | 92 | 26 | 66 | | |
| Lymph node metastasis | | | | 1.669 | 0.196 |
| Yes | 35 | 8 | 27 | | |
| No | 80 | 28 | 52 | | |
| Recurrence | | | | 0.713 | 0.398 |
| Yes | 61 | 17 | 44 | | |
| No | 54 | 19 | 35 | | |
| Distant metastasis | | | | 6.038 | 0.014 |
| Yes | 48 | 9 | 39 | | |
| No | 67 | 27 | 40 | | |
| Vascular invasion | | | | 4.675 | 0.031 |
| Yes | 74 | 17 | 54 | | |
| No | 41 | 19 | 25 | | |

MIBC, muscle-invasive bladder cancer.

*Lentivirus-based RNA interference transfection markedly downregulates B7-H3 mRNA and protein expression of T24 or 5637 cells*. RT-PCR and western blot experiments were used to detect the expression level of B7-H3 mRNA and protein *in vitro*. Silencing of B7-H3 in T24 and 5637 cells was performed by recombinant lentivirus vector LV-B7-H3 shRNA interference transfection. The B7-H3 mRNA and protein expression in B7-H3 shRNA cells was significantly decreased compared to the control cells (Fig. 2). Through this way, we successfully established cancer cells with the silencing of B7-H3 by shRNA.

*Suppression of B7-H3 inhibits cancer cell proliferation by regulating Ki67 and PCNA proteins in vitro*. Colony formation and MTT assay were used to evaluate the effects of B7-H3 silencing on bladder cancer cells. Compared to control cells, stable silencing of B7-H3 in B7-H3 shRNA cells markedly reduced its ability to proliferate *in vitro* (P<0.05; Fig. 3A and B). The above mentioned results suggested that B7-H3 silencing significantly reduced the proliferative potential of cancer cells. Furthermore, to explore the mechanism, we tested the expression level of proliferation-related proteins such as Ki67 and PCNA, and the results showed that the expression of protein Ki67 and PCNA decreased in the B7-H3 shRNA group compared with the control (P<0.05, respectively, Fig. 3C and D). Considered together, our results indicated that B7-H3 silencing could reduce the proliferation by regulating related proteins such as Ki67 and PCNA *in vitro*.

*B7-H3 silencing causes G2 phase arrest*. The flow cytometric analysis was conducted to ascertain whether the anti-proliferative effect was due to cell cycle arrest. Studies showed that significant changes were induced in S and G2 phases of the cell cycle in B7-H3 shRNA cells group compared with control cell group: Accumulation of cells started in sub-G1 phase after 24 h of serum starvation, and DNA accumulation was observed in the G1 phase with a significant increase in cell population in the S phase, which resulted in a significant increase in the cell population in the G2 phase (P<0.05, respectively; Fig. 4).

*B7-H3 silencing inhibits migration and invasion by regulating MMP2 and MMP9 proteins in vitro*. Moreover, wound healing

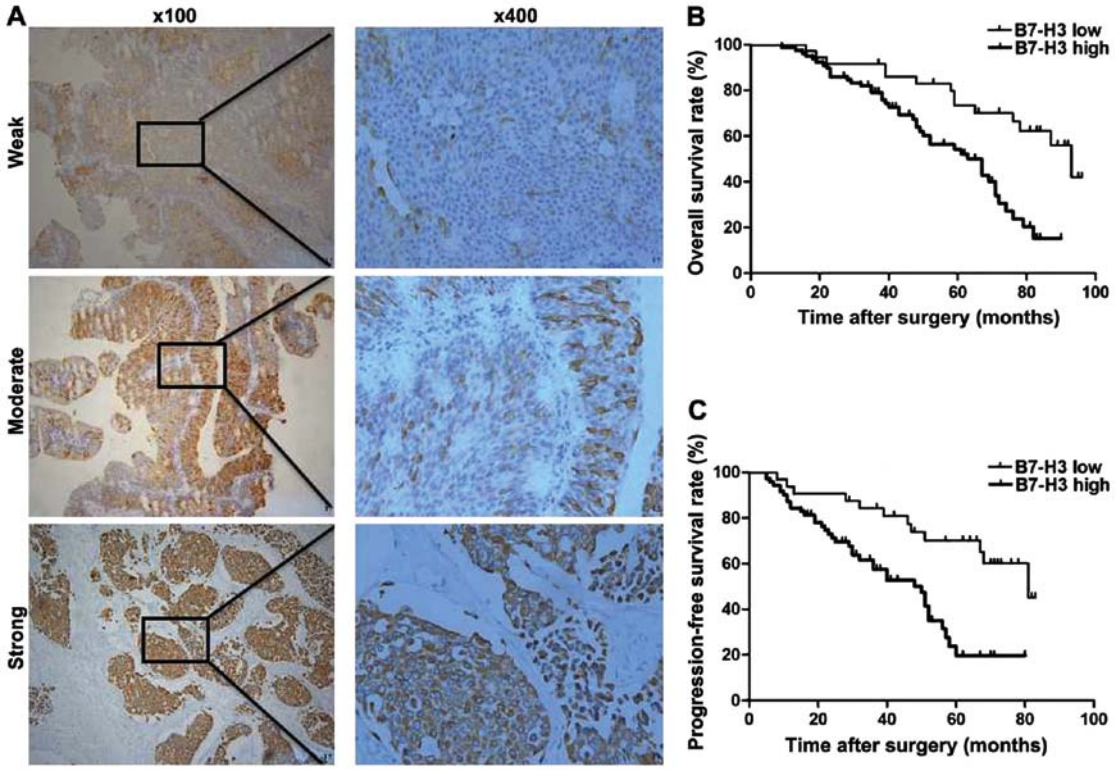


Figure 1. The expression level of B7-H3 in MIBC tissues and the association between B7-H3 expression and OS and PFS in MIBC patients. (A) Weak, moderate, and strong intensity of B7-H3 expressions are shown; various expression levels of B7-H3 protein. (B) Association of OS rates and B7-H3 expression in MIBC patients: Shorter OS was significantly observed in patients with high B7-H3 expression than that in patients with low B7-H3 expression among the 115 MIBC patients (P<0.001). (C) Association of PFS rates with B7-H3 expression in the MIBC patients. Shorter PFS was significantly observed in patients with high B7-H3 expression than that in patients with low B7-H3 expression among the 115 MIBC patients (P<0.001). MIBC, muscle-invasive bladder cancer; B7-H3, B7 homologue 3; OS, overall survival; PFS, progression-free survival.

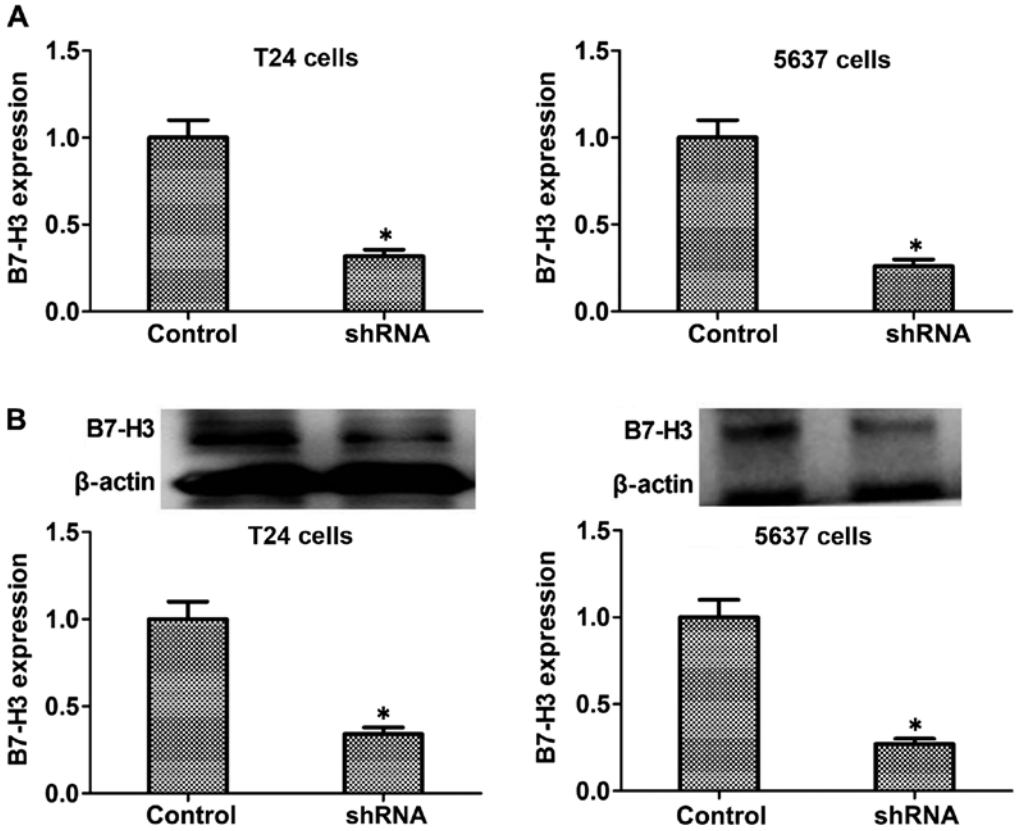


Figure 2. The mRNA and protein expression level of B7-H3 in the established stable cell lines. The mRNA and protein expression level of B7-H3 in the B7-H3 shRNA cells was decreased significantly compared to the control cells (*P<0.05, respectively).

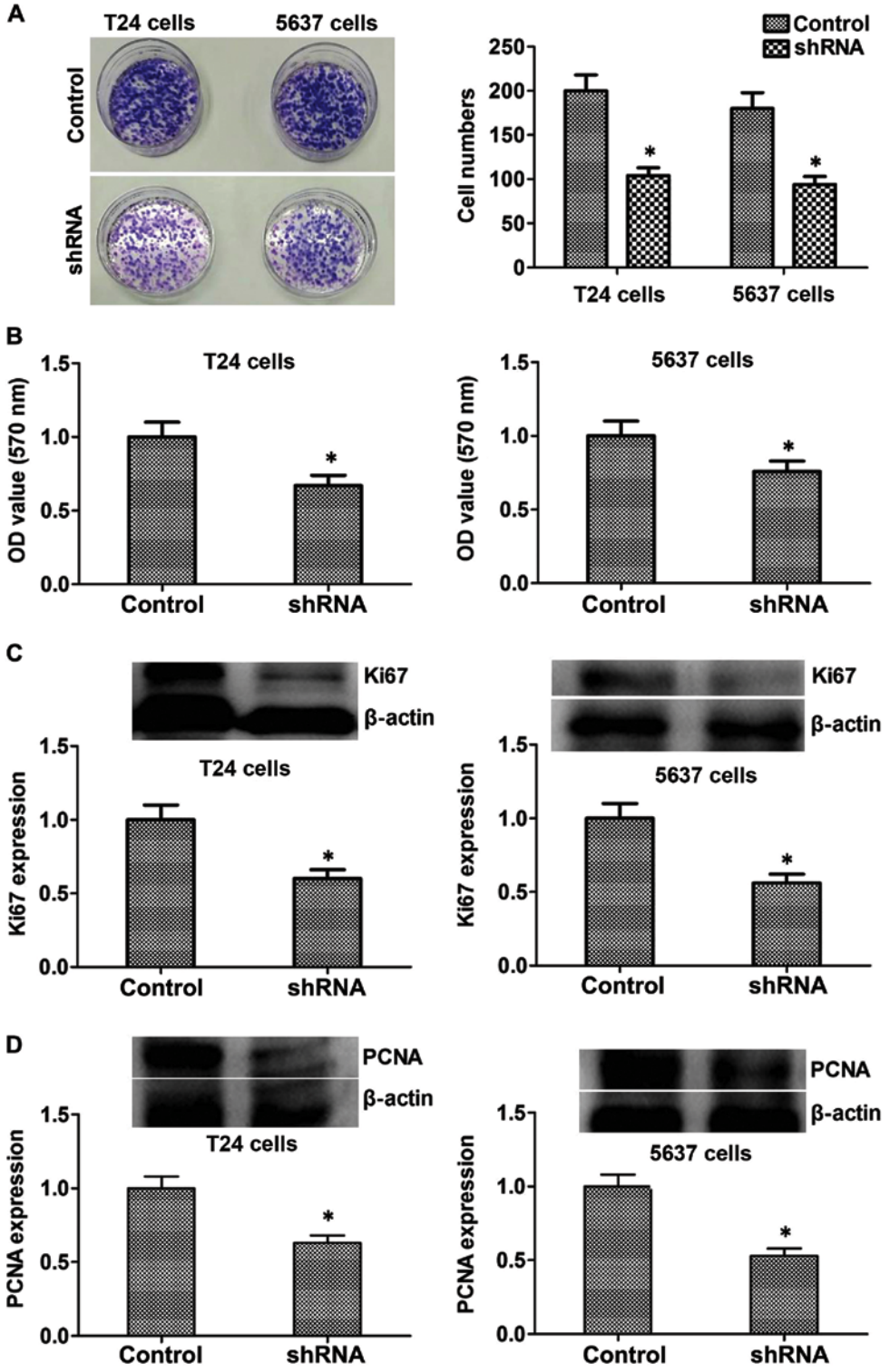


Figure 3. Suppression of B7-H3 inhibits the proliferation of T24 and 5637 cells. (A) Colony formation. The results of the colony formation assay showed decreased colonies in the B7-H3 shRNA group compared to the control *in vitro* (*P<0.05). (B) MTT assay results. The results showed decreased OD value in the B7-H3 shRNA group compared with the control *in vitro* (*P<0.05). (C and D) The results of western blot analysis showed that the expression of the proliferation-related proteins Ki67 and PCNA were significantly decreased in the B7-H3 shRNA group compared with the control group (*P<0.05, respectively).

assay was used to detect changes in cell migration. As shown in Fig. 5A, compared with the control cells, B7-H3 shRNA cells showed larger scratch width (P<0.05). In addition, Transwell assay was used to determine the possible effect of B7-H3 silencing on regulating cancer cell invasiveness. Data showed that the invasive capacity of B7-H3 shRNA cells was decreased compared with the control cells after 48 h (P<0.05; Fig. 5B). Furthermore, to explore the mechanism, we tested the expression level of the proteins which reflect invasion and metastasis such as MMP2 and MMP9, and the results showed that the expression of MMP2 and MMP9 significantly decreased in the B7-H3 shRNA group compared with the control (P<0.05, respectively; Fig. 5C and D). In summary, our results indicated that B7-H3 silencing could reduce the invasiveness of cancer

Table II. Association of overall and progression-free survival and different levels of B7-H3 expression in patients with MIBC (N=115).

| B7-H3 expression | No. of patients | Overall survival % (±SE) | | | P-value | Progression-free survival % (±SE) | | | P-value |
|---|---|---|---|---|---|---|---|---|---|
| | | 1-year | 5-year | 10-year | | 1-year | 5-year | 10-year | |
| High | 79 | 98.7 (1.3) | 14.9 (4.5) | 0 (0.0) | <0.001 | 71.8 (5.1) | 16.8 (4.6) | 0 (0.0) | <0.001 |
| Low | 36 | 100 (0.0) | 58.1 (8.7) | 47.7 (9.9) | | 94.4 (3.8) | 52.0 (9.2) | 43.4 (11.0) | |

MIBC, muscle-invasive bladder cancer.

cells by regulating the related proteins such as MMP2 and MMP9.

*Inhibitory effect of B7-H3 shRNA on MIBC in vivo*. From the results mentioned above, we confirmed that B7-H3 silencing could inhibit proliferation, cause G2 phase arrest and inhibit cell invasion by regulating related proteins *in vitro*. To further demonstrate this, an experiment was performed *in vivo*. After 3 weeks, the tumor volume of the B7-H3 shRNA group was significantly smaller than that found in the control group (P<0.05) (Fig. 6A). Additionally, we evaluated B7-H3 expression of the mouse tumors by western blot analysis, and the results revealed that expression of B7-H3 was reduced due to the knockdown of B7-H3 by shRNA (P<0.05; Fig. 6B). Moreover, we evaluated Ki67 and PCNA expression in the mouse tumors by western blot analysis and immunohistochemistry, and the results suggested that expression of Ki67 and PCNA was also reduced due to the knockdown of B7-H3 by shRNA (P<0.05, respectively, Fig. 6C and D). These results confirmed the inhibitory effect of B7-H3 shRNA *in vivo*.

## Discussion

Bladder cancer is a particularly immunogenic malignancy, and patients with bladder cancer exhibit tumor-associated immunologic suppression (1-7). B7 homologue 3 (B7-H3) is thought to serve as an accessory co-regulator of T-cell response after initial antigen priming (8-15). B7-H3 has been shown to mediate the proliferation of $CD4^+$ and $CD8^+$ T cells and to enhance interferon-γ (IFN-γ) production (8). Moreover, B7-H3 may function as a protective factor in natural killer cell-mediated cytolysis (8-12). However, other studies have suggested that B7-H3 is able to inhibit the proliferation of T cells and reduce the secretion of IFN-γ and interleukin-2 (IL-2) (8-26). Although there is no consensus between the immunological and pathophysiologic roles of B7-H3, aberrant B7-H3 expression has been shown to be closely associated with tumor progression and poor prognosis in human urologic neoplasms (8). The receptor for B7-H3 has not been identified and the precise co-stimulatory and co-inhibitory functions of B7-H3 in immune-regulation remain controversial. A recent review showed that elevated B7-H3 expression is significantly associated with poor survival in many types of cancer (8). Aberrant B7-H3 expression has been shown to be closely associated with tumor metastasis and worse prognosis in several different cancers including non-small cell lung cancer, breast cancer, renal cell cancer, brain cancers (neuroblastoma and glioma), pancreatic cancer, colon cancer, melanoma and prostate cancer (8-31). B7-H3 has been confirmed to promote cancer cell migration and invasion in renal cell cancer and prostate cancer (8,13,14). Clinical data indicate that B7-H3 may be exploited as a co-inhibitory molecule in immune evasion process (8-20). Some scholars speculate that the biological role of B7-H3 may differ from one tumor type to another and changes during disease progression. However, little is known about the exact impact of B7-H3 expression in bladder cancer, and conflicting opinions on the role of B7-H3 expression in bladder cancer are still being debated (11,15). As known, bladder cancer is a heterogeneous disease that spans a broad spectrum from non-muscle-invasive bladder cancer (NMIBC)

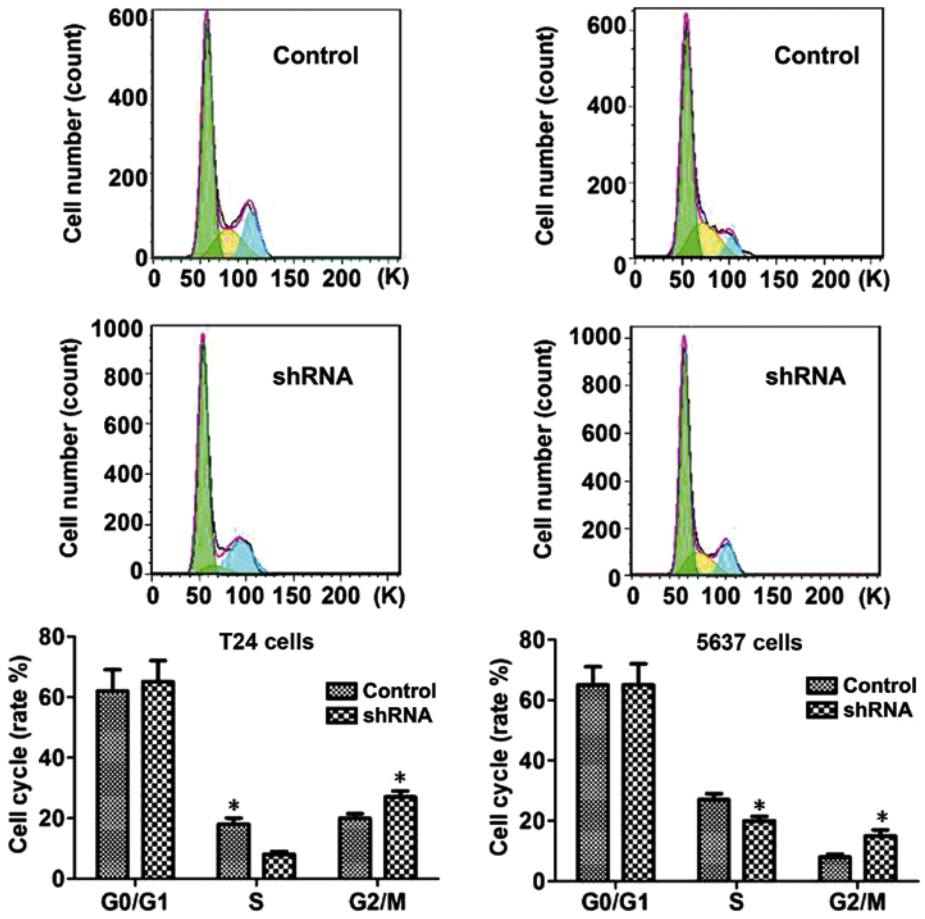


Figure 4. Influence of B7-H3 on the cell cycle of T24 and 5637 cells. The significant increase in the cell population in the S phase and a significant increase in the cell population in the G2 phase were observed (*P<0.05, respectively).

to muscle-invasive bladder cancer (MIBC) (16). Conclusions from the previous research usually depend on the study of a mixed cohort including the two types of bladder cancer patients, for which the lack of separate analysis of subgroups of MIBC or NMIBC exist. Considering that the risk of tumor invasion and metastasis with MIBC or NMIBC is quite different, the original intention of our study is to focus on the independent analysis of MIBC patients and investigate whether there are different findings, at least partly, from previous reports. We examined immunohistochemical expression of B7-H3 in clinical specimens from 115 MIBC patients and evaluated the associations between B7-H3 expression, clinicopathological features and outcomes. Notably, the present study showed that MIBC patients with high B7-H3 expression were more likely to manifest advanced tumor stage and a higher proportion of distant metastasis, whose OS and PFS were significantly shorter than patients with low B7-H3 expression. These different results with other studies provide a hypothesis of the function of B7-H3 at the clinical level, perhaps through facilitating tumor progression of bladder cancer, especially in MIBC. Recent studies demonstrated that B7-H3 expression in tumor cells was correlated with malignant behaviors, such as proliferation, invasion and metastatic potential which finally contributes to cancer progression (8,17,18). In order to verify our clinical findings, we transfected human bladder cancer T24 and 5637 cells with targeted silencing of B7-H3, using lentivirus-based RNA stable interference transfection.

There has been previous research to confirm the role of B7-H3 in migration and invasion. Some studies indicate that B7-H3 silencing reduces the expression of metastasis-associated proteins such as matrix metalloproteinase (MMP)-2, MMP-9, signal transducer and activator of transcription 3 (STAT3) and the level of secreted interleukin-8 (IL-8) (8,18,19,31). An additional mechanism suggests that B7-H3 also influences liver cancer aggressiveness and invasiveness through the JAK3/STAT3/SLUG signaling pathway (19). In hepatocellular carcinoma, B7-H3 has been found to promote epithelial-mesenchymal transition (EMT), and STAT3 has also been found to induce NF-κB activity, which influences the expression of IL-8, VEGF and MMPs in the tumor microenvironment (8,30). Xie *et al* reported that B7-H3 increases the activity of NF-κB signaling, which stimulated the *in vitro* and *in vivo* invasion of pancreatic cancer cells (21). It has also been reported that B7-H3 promoted cell migration and invasion through the JAK3/STAT3/MMP9 signaling pathway in colorectal cancer (31). Consistent with these results, our results demonstrated that B7-H3 could promote cell migration and invasion *in vitro*. In addition, we confirmed that B7-H3 promoted cell proliferation, and inhibited G2 phase arrest *in vitro*. Furthermore, our results showed that B7-H3 silencing could alter the T24 and 5637 cell behaviors by regulating their related proteins. However, this finding still needs to be confirmed in other types of MIBC cells and in different types of cancer. The central key is investigation of the

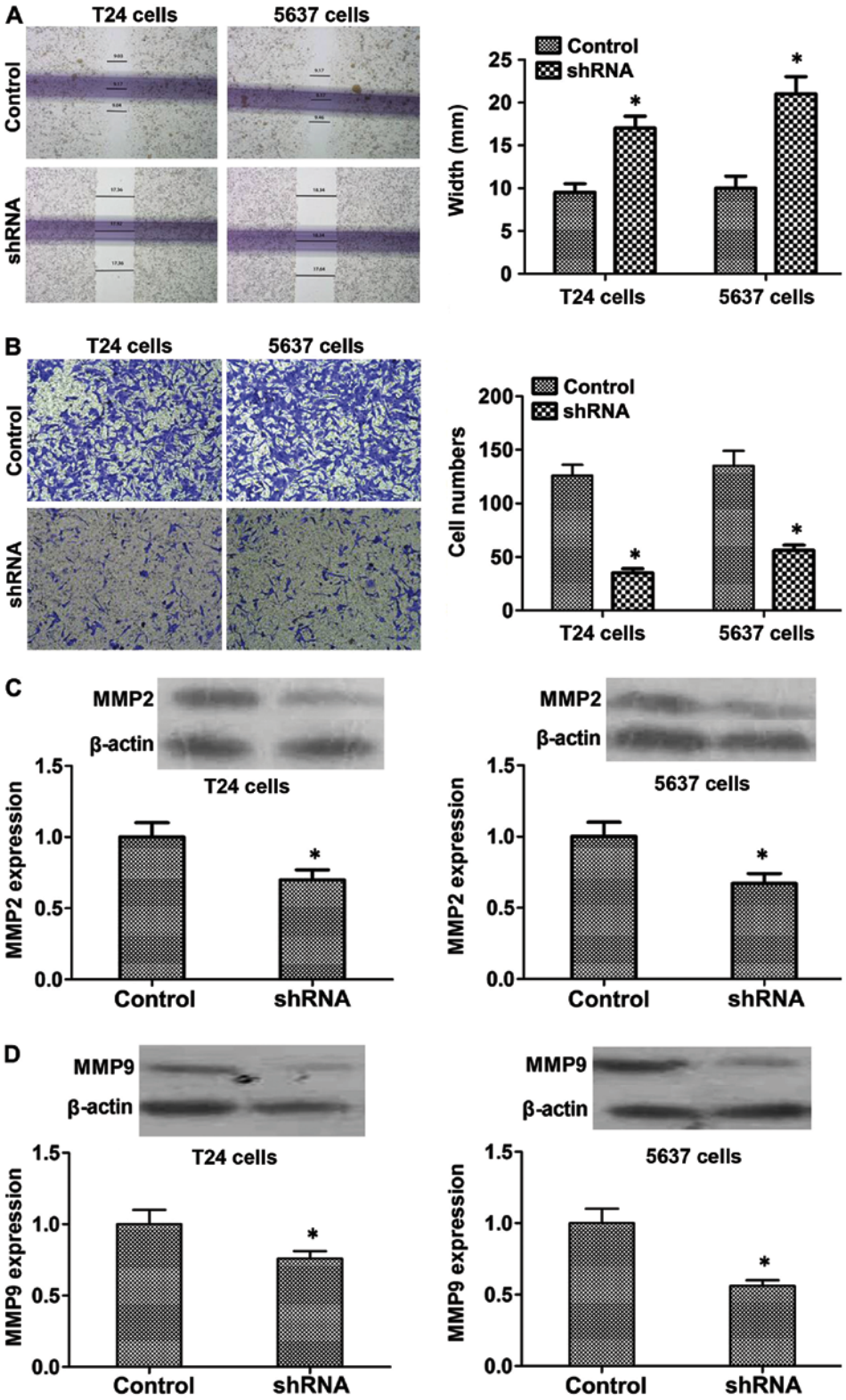


Figure 5. Suppression of B7-H3 inhibits migration and invasion by MMP2 and MMP9 signaling. (A) Scratch assay. The results showed that a significantly larger scratch width in the T24/B7-H3 shRNA group when compared with the control in the T24 cells (*P<0.05). (B) Transwell assay. The results showed significantly less cell numbers in the T24/B7-H3 shRNA group compared with that in the control in the T24 cells (*P<0.05). Results are representative of at least 3 separate experiments. (C and D) The results of western blot analysis showed that the expression levels of the metastatic-related proteins MMP2 and MMP9 were significantly decreased in the T24/B7-H3 shRNA group compared with the control group (*P<0.05, respectively).

signaling pathways and molecular mechanisms, which is the next step underway in future research. Finally, we confirmed similar results *in vivo* before clinical experiments. Recently, a study reported that B7-H3 could promote the migration and invasion of bladder cancer cells via the PI3K/Akt/STAT3 signaling pathway (32). Although they found that B7-H3 did not affect cell proliferation *in vitro*, which was different from ours, their results proved that B7-H3 had an effect on the

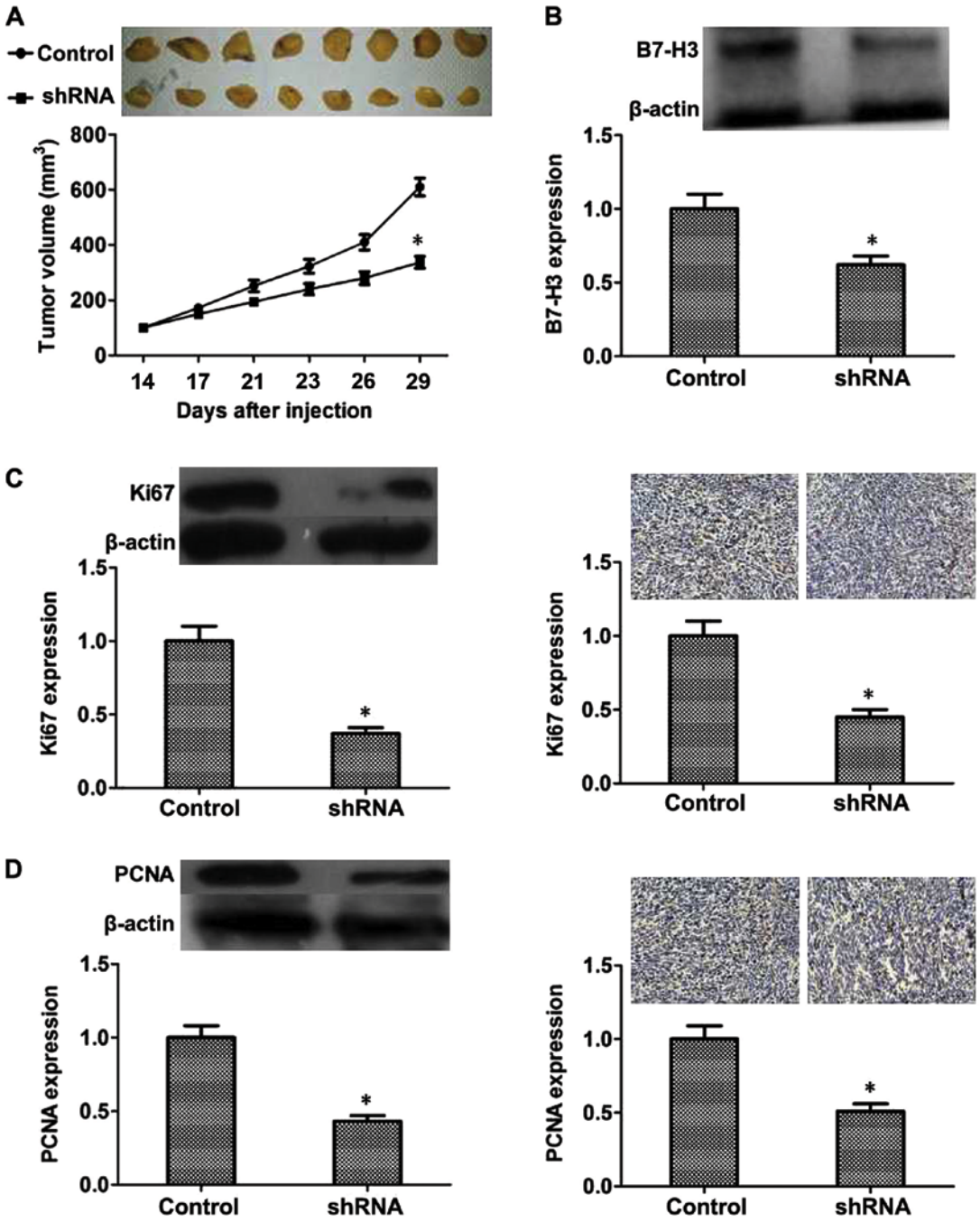


Figure 6. Inhibitory effect of B7-H3 shRNA on MIBC *in vivo*. (A) Tumor volumes in the different groups. After 29 days of treatment, the tumor volume in the B7-H3 shRNA group was smaller than that in the control group (*P<0.05). (B) Western blot analysis results of B7-H3 expression in the two different groups. B7-H3 expression was decreased in the B7-H3 shRNA group in the T24 cell-derived tumor xenografts in the nude mice (*P<0.05). (C and D) The results of western blot analysis and immunohistochemistry showed that the expression of Ki67 and PCNA expression in the mouse tumors was decreased in the T24/B7-H3 shRNA group compared with the control group (*P<0.05, respectively).

PI3K/Akt/STAT3 signaling pathway which could influence proliferation. Our results did confirm the function of B7-H3 on the proliferation by regulating Ki67 and PCNA, and we also proved this effect *in vivo*. In addition, we demonstrated that suppression of B7-H3 significantly caused G2 phase arrest. Therefore, our results should be reliable and more studies are needed to explore the complicated mechnism.

Moreover, B7-H3 expression has been reported in kidney cancer, endothelium of colon, lung, and breast cancers (27-31). In addition, B7-H3 has been detected in tumor vasculature, and influenced the cancer microenvironment as soluble B7-H3 (sB7-H3) in pancreatic cells, which leads to a significant increase in migration and invasion (21,30). B7-H3 has also been proven to lead to an upregulation of NF-κB through a TLR4-dependent mechanism, which leads to a significant increase in VEGF and IL-8 expression, resulting in tumor invasion and angiogenesis (21,29).

Considered together, these series of cellular-function experiments were confirmed with our clinical findings in the immunohistochemistry assay. The expression of B7-H3 protein in tumor tissues and T24 or 5637 cells indicates its clinical relevance in MIBC. Our study provided a further investigation

and understanding of the B7-H3 molecular mechanisms which were involved in the proliferation and metastatic potential of MIBC. All the results demonstrated that B7-H3 may play a pro-tumor role in MIBC progression and could be regarded as an immuno-therapeutic target for MIBC patients.

In conclusion, we first investigated the expression of co-stimulatory molecule B7-H3 in MIBC tumor specimens, and we found that patients with high B7-H3 expression manifested high malignant progression and poor prognosis in MIBC. Then we demonstrated that B7-H3 could promote the development of MIBC *in vitro* and *in vivo*. Thus, B7-H3 may be a potential novel biomarker for MIBC.

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**Availability of data and materials**

The dataset supporting the conclusions of this article is included within the article. Raw data of the present study are available from the corresponding author upon reasonable request.

**Authors' contributions**

ZLX and YZ carried out the experiment of molecular biology and drafted the manuscript. ZLX and LW participated in the sequence alignment. ZLX, YZ, LW and HWM participated in the design of the study and performed the statistical analysis. ZLX, PFL and BES conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

Human samples and animals in this study were approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. The research involving human participants was approved by the Fourth Hospital of Hebei Medical University and the equivalent committee. The participants provided written informed consent before enrollment in this study. The consent forms were not be provided in this article due to the large number and they were all written in the Chinese language. The animal study was conducted with approval of the Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal surgery was performed with care to alleviate pain.

**Patient consent for publication**

The participants provided written informed consent before enrollment in this study.

**Competing interests**

The authors declare that they have no competing interests.

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