RRBP1 is highly expressed in bladder cancer and is associated with migration and invasion

MINGSHUAI WANG[1], SAI LIU[1], BOLIN ZHOU[1], JIANWEN WANG[1], HAO PING[2] and NIANZENG XING[3]

[1]Department of Urology, Institute of Urology, Beijing Chaoyang Hospital, Capital Medical University, Beijing 100020; [2]Department of Urology, Beijing Tongren Hospital, Capital Medical University, Beijing 100730; [3]Department of Urology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, P.R. China

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Abstract. Ribosome-binding protein 1 (RRBP1) is a marker for colorectal, lung, esophageal and prostate cancer. However, the association between RRBP1 and bladder cancer is not completely understood. The present study aimed to evaluate the expression and function of RRBP1 in bladder cancer. The association between RRBP1 expression and clinicopathological characteristics, as well as the prognosis of bladder cancer was analyzed. RRBP1 expression was further analyzed in bladder cancer cell lines via reverse transcription-quantitative PCR and western blotting. RRBP1 knockdown was established using short hairpin RNAs to investigate the function of RRBP1 in T24 cells. Compared with healthy bladder tissue, RRBP1 expression levels were significantly upregulated in bladder cancer tissue. High RRBP1 expression was associated with tumor stage, lymph node metastasis and shorter overall survival time. RRBP1 protein was highly expressed in bladder cancer cell lines compared with normal SV-HUC-1 cells. Compared with the control group, RRBP1 knockdown inhibited T24 migration and invasion by downregulating the expression of C-C chemokine receptor type 7 (CCR7) protein. In conclusion, the present study indicated that RRBP1 was associated with bladder cancer migration, invasion and prognosis, and CCR7 might serve a role in the process.

Correspondence to: Professor Hao Ping, Department of Urology, Beijing Tongren Hospital, Capital Medical University, 1 Dongjiao Mingang, Dongcheng, Beijing 100730, P.R. China
E-mail: pinghaocy@163.com

Professor Nianzeng Xing, Department of Urology, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 17 Beijing Hua Wei South Road, Pan Jia Yuan Nan Li Block, Beijing 100021, P.R. China
E-mail: xingnianzeng@hotmail.com

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Introduction

Bladder cancer is one of the most common genitourinary malignancies in the world (1). Complete resection is the primary treatment strategy for patients with bladder cancer; however, a study in Germany reported that the 5-year overall survival rate of patients with bladder cancer after radical cystectomy was <60% (2).

Ribosome-binding protein 1 (RRBP1) is an endoplasmic reticulum (ER) membrane protein that is critical for ribosome binding and the transportation of nascent proteins (3). RRBP1 inhibition could cause ER stress and significantly reduce cell tumorigenicity (4) RRBP1 was reported to be overexpressed in lung, colorectal, endometrial and prostate cancer (4-7). High RRBP1 expression could promote esophageal and colorectal cancer progression, and can be used to predict patient prognosis (5,8). However, to the best of our knowledge, the expression of RRBP1 in bladder cancer has not been previously reported.

C-C chemokine receptor type 7 (CCR7) is a cell surface chemokine receptor (9). Increasing evidence has demonstrated that CCR7 is involved in the development and progression of bladder (10), lung (11), breast (12) and colorectal (13) cancer. Therefore, it was hypothesized that downregulation of RRBP1 may reduce CCR7 expression leading to inhibition of bladder cancer migration and invasion.

The present study aimed to investigate the role of RRBP1 in bladder cancer. The Cancer Genome Atlas (TCGA) database was searched and analyzed to compare RRBP1 mRNA expression levels between bladder cancer and healthy tissues, and the overall survival according to these levels. RRBP1-knockdown was used to analyze the expression levels of RRBP1 and different cell functions in bladder cancer cell lines. Finally, the association between RRBP1 and CCR7 was investigated by comparing CCR7 expression between control and RRBP1-knockdown cells.

Materials and methods

Bioinformatics analysis. RRBP1 mRNA expression and overall survival in bladder cancer tissues and healthy tissues in TCGA were analyzed using the University of Alabama

Cancer Database (ualcan.path.uab.edu/analysis.html) (14). Subsequently, RRBP1 mRNA expression at different tumor and lymph node stages was analyzed using LinkedOmics (www.linkedomics.org/admin.php) (15).

Immunohistochemistry (IHC). The present study was approved by the Ethics Committee of Beijing Chaoyang Hospital (approval no. 2020-3-18-85). All subjects provided written informed consent after oral consent to participate in the present study. The clinical data of the patients are presented in Table SI. All surgical specimens were fixed with 10% formalin (10:1 ratio of formalin to tissue) at room temperature for 24 h and then embedded in paraffin. The bladder cancer tissues were pathologically confirmed and para-carcinoma tissues were obtained ~2 cm from the tumor margin. The section thickness was 3-4 μm. Deparaffinization and rehydration were performed according to standard procedures (16). After blocking with 5% bovine serum albumin (cat. no. G5001; Wuhan Servicebio Technology Co., Ltd.) at room temperature for 30 min, sections were incubated overnight at 4˚C with an anti-RRBP1 primary antibody (1:500; cat. no. ab95983; Abcam) and then an HRP-conjugated secondary antibody (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 50 min at room temperature. Chromogen detection was performed using DAB reagent (cat. no. G1211; Wuhan Servicebio Technology Co., Ltd.) for 3-5 min. Subsequently, the slices were rinsed with running water and counterstained with haematoxylin (Wuhan Servicebio Technology Co., Ltd.) for 3 min at room temperature. The expression levels were evaluated by two pathologists blinded to the clinical characteristics of the patients according to proportion of cell staining (0, 0%; 1, ≤25%; 2, 26-50%; 3, 51-75%; and 4, >75%) and the staining intensity (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) using a light microscope (magnification, x400). The final score was calculated by multiplying the cell staining proportion score and the staining intensity score. If the final score was >9, protein expression was considered high, but if the final score was ≤8, protein expression was considered low.

Cell lines and cell culture. Bladder cancer cell lines (5637, T24 and UM-UC-3) and an immortalized urothelial cell line (SV-HUC-1) were obtained from China Infrastructure of Cell Line Resources, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. All cells were cultured at 37˚C with 5% CO_2 and 95% O_2. SV-HUC-1 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (both HyClone; Cytiva). 5637, T24 and UM-UC-3 cells were cultured in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% FBS (HyClone; Cytiva), 100 U/ml penicillin G and 100 μg/ml streptomycin (Lonza Group, Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (cat. no. 3101-100; Shanghai Pufei Biotechnology Co., Ltd.). Total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (cat. no. M1705; Promega Corporation). The Oligo dT was provided by Sangon Biotech Co., Ltd. (cat. no. B0205) and the dNTPs (10 mM) were provided by Promega Corporation (cat. no. U1240). After heat treatment of RNA/primer mixture at 70˚C for 10 min and cooling immediately on ice for 10 min, the RT reaction was processed at 42˚C for 1 h and 70˚C for 10 min. Subsequently, qPCR was performed using ExTaq (Takara Bio, Inc.). The SYBR Master Mixture was provided by Takara Bio, Inc. (cat. no. DRR041B). The following primer were used for the qPCR: RRBP1 forward, 5'-GAGATGGCGAAAACTCACCAC-3' and reverse, 5'-CTCGAAGGAGGACAGTCACAT-3'; CCR7 forward, 5'-TTCATCGGCGTCAAGTTCC-3' and reverse, 5'-AAGGTGGTGGTGGTCTCG-3'; and GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 30 sec; followed by 40 cycles of 95˚C for 5 sec; and final extension at 60˚C for 30 sec. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (17) and normalized to the internal reference gene GAPDH.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Technology). Protein concentration was determined using a BCA Protein Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology). Proteins (50 μg) were separated via 10% SDS-PAGE and transferred to PVDF membranes, which were blocked with 5% non-fat milk powder. The membranes were incubated overnight at 4˚C with primary antibodies targeted against the following: RRBP1 (1:500; cat. no. ab95983; Abcam), CCR7 (1:1,000; cat. no. ab32527; Abcam) and GAPDH (1:2,000; cat. no. ab8245; Abcam). Subsequently, they were incubated with HRP-conjugated anti-rabbit IgG (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) and anti-mouse IgG (1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibodies. Protein bands were visualized using an ECL kit (cat. no. M3121/1859022; Thermo Fisher Scientific, Inc.). GAPDH was used as the loading control. Image J (version 1.51n; National Institutes of Health) was used to quantify blots.

Lentivirus construction and infection. Lentivirus RRBP1 short hairpin RNA (shRNA) and negative control shRNA both containing GFP were purchased from Shanghai GeneChem Co., Ltd. The target sequences of the shRNAs were as follows: shRNA-1, 5'-GCAAGCCAGGATGGATATTTA-3'; shRNA-2, 5'-GCCAAGAAGAAGTCTGGTTCA-3'; and negative control shRNA, 5'-TTCTCCGAACGTGTCACGT-3'. T24 cells were seeded in a six-well plate at 30% confluence, the medium was removed and replaced with 2 ml complete medium containing Polybrene (cat. no. sc-134220; Santa Cruz Biotechnology, Inc.) at a final concentration of 5 μg/ml. T24 cells were transfected with negative control or RRBP1 shRNA (MOI=10) according to the manufacturer's instructions (Shanghai GeneChem Co., Ltd). The volume and concentration of control, shRNA-1 and shRNA-2 lentivirus were 2 μl $1x10^9$ transduction units (TU)/ml, 3.33 μl $6x10^8$ TU/ml and 3.33 μl $6x10^8$ TU/ml, respectively. After 16 h of infection, the medium was changed to complete medium. At 72 h post-transfection, transfection efficiency was assessed via RT-qPCR and western blotting.

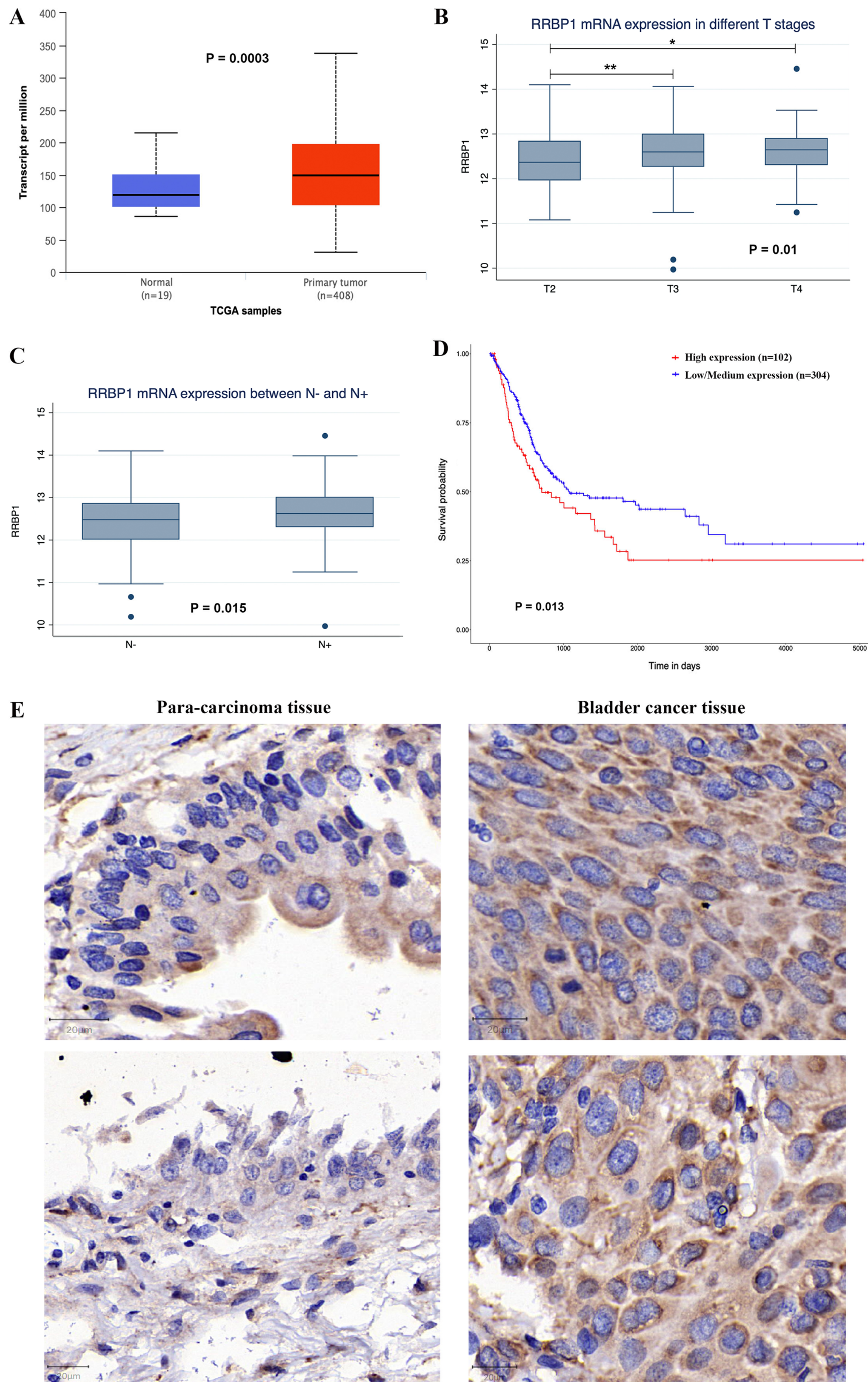

Figure 1. RRBP1 is upregulated in human bladder cancer and is associated with overall survival. (A) RRBP1 mRNA expression levels in tumor tissues and healthy tissues obtained from patients with bladder cancer (P=0.0003). (B) RRBP1 expression levels in different T stages (P=0.01). (C) RRBP1 expression levels according to positive or negative lymph node metastasis (P=0.015). (D) RRBP1 expression and overall survival rate (P=0.013). (E) Representative images of IHC in bladder cancer and para-carcinoma tissues (magnification, x400). *P<0.05; **P<0.01. RRBP1, ribosome-binding protein 1; TCGA, The Cancer Genome Atlas; T, tumor; N, lymph node.

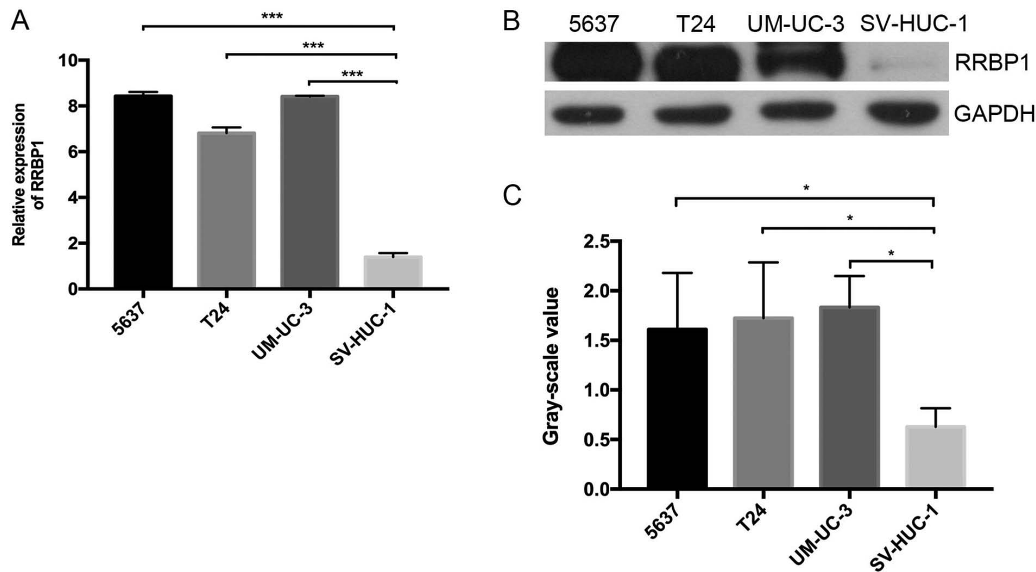

Figure 2. RRBP1 expression in different bladder cancer cell lines. (A) RRBP1 mRNA expression levels in 5637, T24, UM-UC-3 and SV-HUC-1 cells. RRBP1 protein expression levels in 5637, T24, UM-UC-3 and SV-HUC-1 cells were determined by (B) western blotting and (C) its semi-quantification. *P<0.05; ***P<0.001. RRBP1, ribosome-binding protein 1.

Migration and invasion assays. Cell invasion was assessed using Matrigel-coated Transwell chambers (cat. no. 354480; Corning Inc.) and cell migration was assessed using Transwell chambers. Cells were seeded (1x10^5 cells/well) in DMEM without FBS into the upper chamber. DMEM (600 μl) supplemented with 30% FBS was plated into the lower chambers. Following incubation at 37˚C for 16 h, non-invading/migratory cells were removed using cotton swabs and invading/migratory cells were fixed with 4% methanol for 30 min at room temperature. Following staining with 0.5% crystal violet at room temperature for 5 min, invading/migratory cells were counted using a light microscope (magnification, x100 and x200).

For the wound healing assay, T24 cells (5x10^4) were seeded into a 96-well plate and at 90% confluence, a single scratch was made in the middle of each well. GFP-transfected cells were incubated in DMEM supplemented with 1% FBS at 37˚C. Cell migration was observed using a Celigo Image cytometer (Nexcelom Bioscience LLC) at 0, 8 and 24 h, and the migration distance was automatically measured. The Celigo Image cytometer identified and scanned GFP-positive cells. The images captured by the image cytometer were equivalent to 100x the same resolution of the microscope.

Statistical analysis. Statistical analyses were performed using Stata software (version 14.0; StataCorp LP) and GraphPad Prism software (version 7; GraphPad Software, Inc.). Data are presented as the mean ± SD. The unpaired Student's t-test was used to analyze comparisons between two groups. The Kruskal-Wallis test was used to analyze comparisons between different tumor stages and lymph node metastasis status, followed by the Nemenyi test for individual comparisons among multiple groups of tumor stages. One-way ANOVA with Bonferroni test was used to analyze comparisons among multiple groups in the cell experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

RRBP1 is highly expressed in bladder cancer and is correlated with patient prognosis. To investigate whether RRBP1 served a role in bladder cancer, the TCGA database (14) was searched and LinkedOmics was used to analyze multi-omics data (15). RRBP1 expression was significantly upregulated in bladder cancer tissues compared with healthy bladder tissues (P=0.0003; Fig. 1A). High RRBP1 expression was associated with tumor stage (P=0.01) and lymph node metastasis (P=0.015; Fig. 1B and C). The IHC results indicated that RRBP1 protein expression was higher in bladder cancer tissues compared with para-carcinoma tissues (Figs. 1E and S1). Moreover, patients with high RRBP1 expression displayed shorter overall survival compared with patients with low and medium RRBP1 expression in the UALCAN database (P=0.013; Fig. 1D).

RRBP1 overexpression in bladder cancer cells. RRBP1 expression was also detected in bladder cancer cell lines (5637, T24 and UM-UC-3) and SV-HUC-1 cells via RT-qPCR. SV-HUC-1 is an epithelial SV40 cell line that originated from the ureter. RRBP1 mRNA expression was significantly higher in 5637 (P<0.01), T24 (P<0.01) and UM-UC-3 cells (P<0.01) compared with SV-HUC-1 cells (Fig. 2A). Similarly, RRBP1 protein expression levels were significantly higher in bladder cancer cell lines compared with SV-HUC-1 cells (P<0.05; Fig. 2B and C). The results indicated that RRBP1 was highly expressed in bladder cancer, which may indicate that it serves a role in tumorigenesis.

RRBP1 knockdown in a high-expressing bladder cancer cell. T24 cells were infected with RRBP1 shRNA-containing lentiviruses, shRNA-1 or shRNA-2. RRBP1 knockdown efficiency was assessed via western blotting and RT-qPCR. Compared with the control group, shRNA-1 significantly inhibited

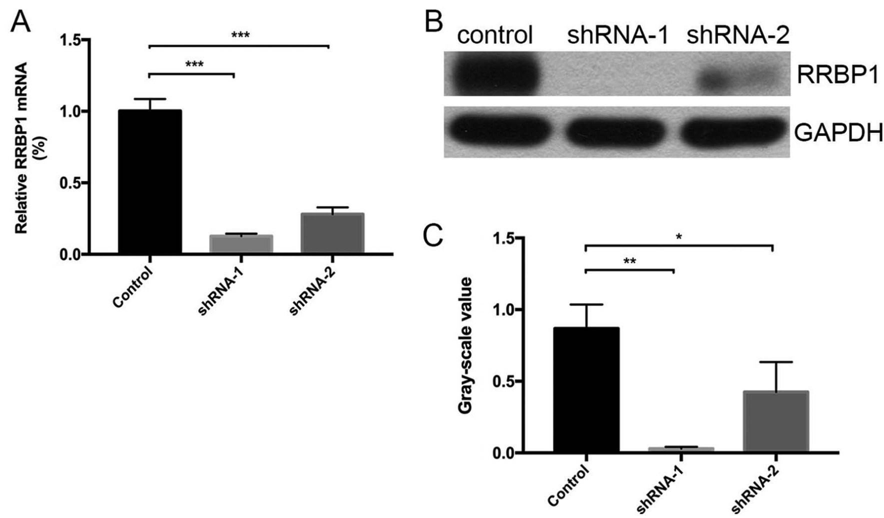

Figure 3. shRNA-1 and shRNA-2 knock down RRBP1 expression in T24 cells. (A) RRBP1 mRNA expression levels following RRBP1 knockdown. RRBP1 protein expression levels following RRBP1 knockdown were (B) determined by western blotting and (C) semi-quantified. *P<0.05; **P<0.01; ***P<0.001. shRNA, short hairpin RNA; RRBP1, ribosome-binding protein 1.

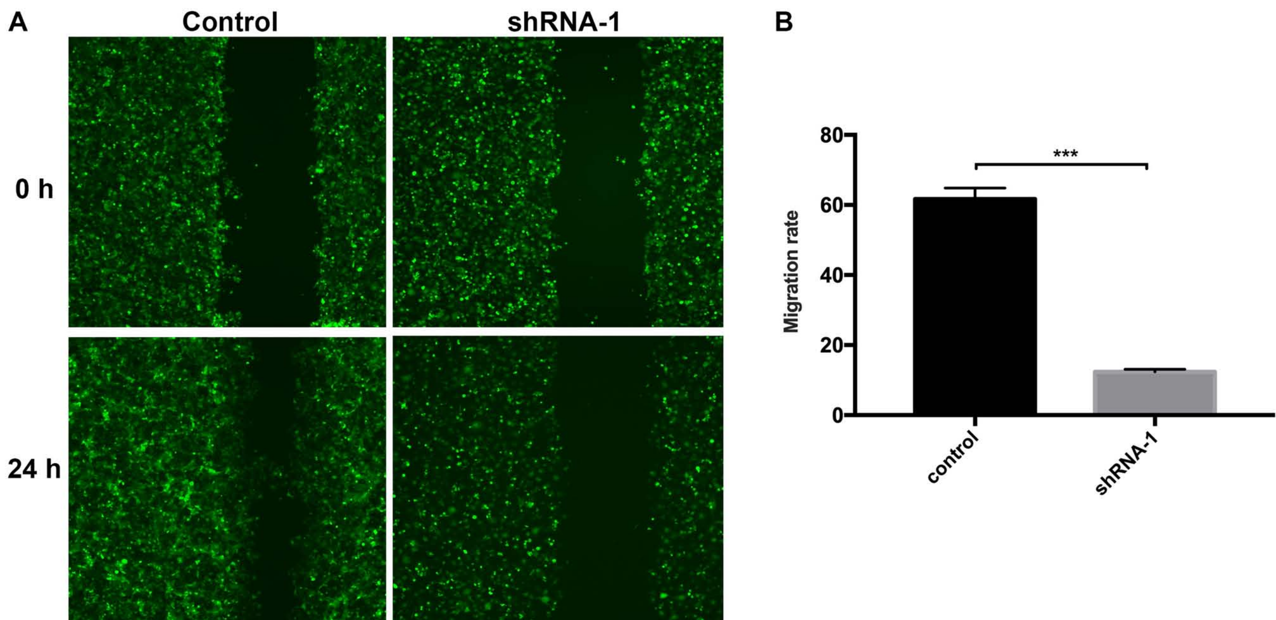

Figure 4. RRBP1 knockdown inhibits T24 cell migration. (A) Representative images of the wound healing assay (magnification, x100). (B) Quantification of T24 cell migration. ***P<0.001. RRBP1, ribosome-binding protein 1; shRNA, short hairpin RNA.

RRBP1 expression by 87.3% at the mRNA level (P<0.001) and by 96.55% at the protein level (P=0.002), whereas shRNA-2 significantly inhibited RRBP1 expression by 72.0% at the mRNA level (P<0.001) and by 50.57% at the protein level (P=0.038) (Fig. 3).

RRBP1 knockdown impairs T24 cell migration and invasion. Subsequently, the effect of RRBP1 on T24 cell migration and invasion was assessed. The wound healing assay indicated that T24 cell migration was significantly reduced following shRNA-1 transfection compared with the control group (Fig. 4). Similarly, the Transwell assay results suggested that RRBP1 knockdown by shRNA-1 significantly inhibited T24 cell migration compared with the control group (Fig. 5A and B). In addition, RRBP1 knockdown by shRNA-1 significantly decreased T24 cell invasion compared with the control group (Fig. 5A and B).

RRBP1 knockdown suppresses T24 cell migration and invasion via suppression of CCR7. Previous studies indicated that CCR7 could enhance bladder cancer proliferation, migration and invasion (18-20). Therefore, the association between RRBP1 and CCR7 was investigated. Compared with the control group, the mRNA expression levels of CCR7 were significantly increased after RRBP1 knockdown (P=0.007 vs. shRNA-1 and P=0.036 vs. shRNA-2; Fig. 6A); however, the protein expression levels of CCR7 were significantly decreased after RRBP1 knockdown (P<0.05; Fig. 6B and C). The results indicated that RRBP1 knockdown might suppress CCR7

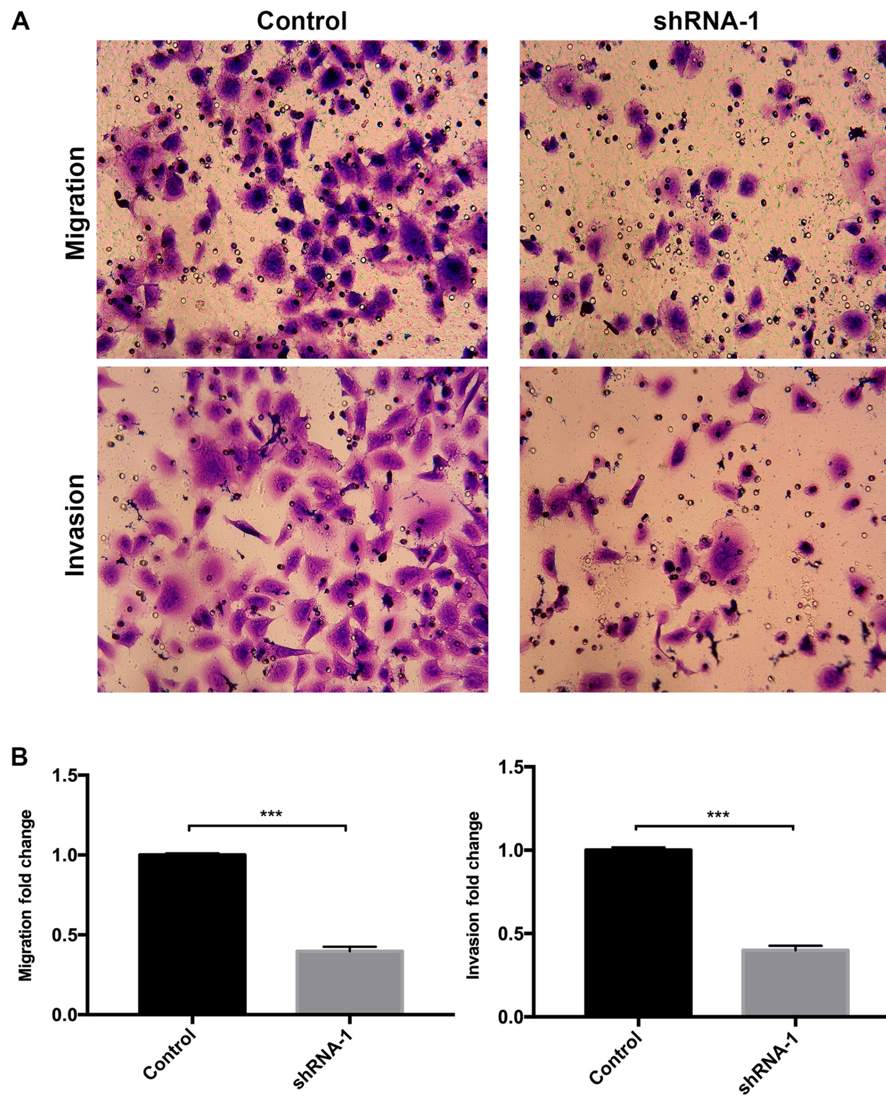

Figure 5. T24 cell migration and invasion are inhibited by RRBP1 knockdown. (A) Representative images of the Transwell assay (magnification, x200). (B) Quantification of T24 cell migration and invasion. ***P<0.001. RRBP1, ribosome-binding protein 1; shRNA, short hairpin RNA.

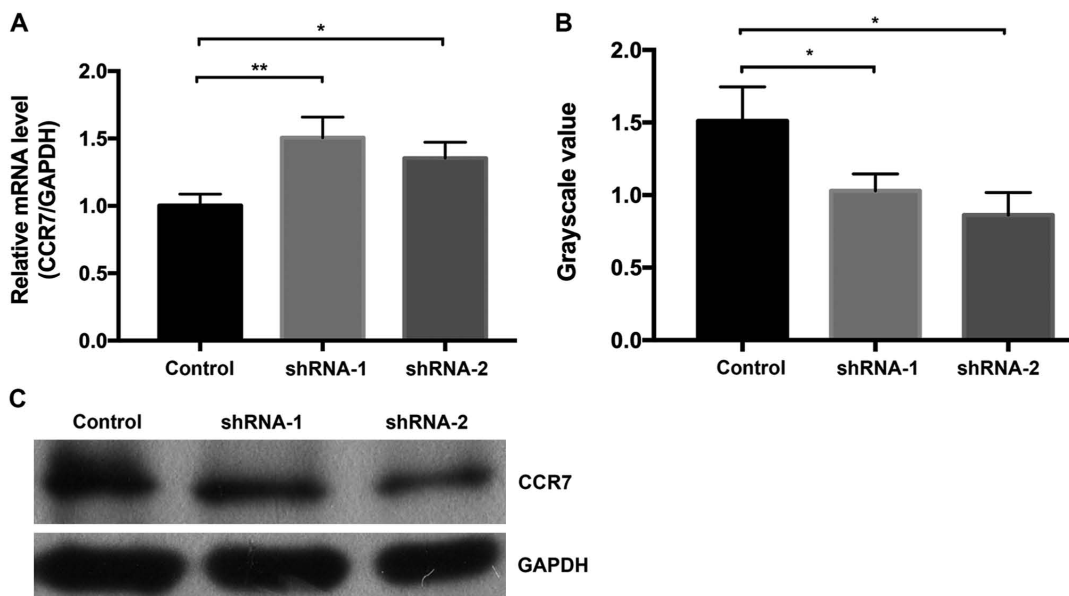

Figure 6. CCR7 expression following RRBP1 knockdown in T24 cells. CCR7 (A) mRNA and (B) protein expression following RRBP1 knockdown. (C) CCR7 protein expression levels were determined by western blotting. *P<0.05; **P<0.01. CCR7, C-C chemokine receptor type 7; RRBP1, ribosome-binding protein 1.

mRNA translation and inhibit bladder cancer cell migration and invasion.

Discussion

To the best of our knowledge, the present study was the first to investigate RRBP1 expression in bladder cancer. To investigate the association between RRBP1 expression and bladder cancer, the TCGA database was searched. RRBP1 mRNA expression was significantly higher in bladder cancer tissues compared with healthy bladder tissues, and RRBP1 expression was associated with tumor stage, lymph node metastasis and overall survival. RRBP1 was also upregulated in bladder cancer cells compared with normal urothelial cells. RRBP1 knockdown inhibited bladder cancer cell migration and invasion compared with the control group. CCR7 mRNA expression levels were significantly increased following RRBP1 knockdown compared with the control group. By contrast, the expression of CCR7 protein was significantly decreased by RRBP1 knockdown compared with the control group, which indicated that RRBP1 knockdown might suppress CCR7 mRNA translation and inhibit bladder cancer cell migration and invasion.

A number of studies have demonstrated that RRBP1 overexpression correlated with the progression and prognosis of various types of cancer. Pan *et al* (5) examined RRBP1 expression via IHC in colorectal tissues and reported that RRBP1 was aberrantly overexpressed in colorectal cancer. Compared with patients with low RRBP1 expression, patients with high RRBP1 expression had shorter disease-specific survival. RRBP1 was also highly expressed in esophageal carcinoma tissues and was associated with T stage, lymph node metastasis, TNM stage and survival (8). Similar studies were performed in endometrial endometrioid adenocarcinoma and prostate cancer, and RRBP1 was recognized as a potential protein marker (6,7). However, the association between RRBP1 and bladder cancer is not completely understood. In the present study, RRBP1 was upregulated in bladder cancer tissues compared with para-carcinoma tissues, which was associated with bladder cancer cell migration and invasion.

RRBP1 was originally identified as a ribosome-binding protein on the rough ER. RRBP1 has been studied in yeast, where it is a member of the ER stress response and associated unfolded protein response (UPR) (21). shRNA-mediated RRBP1 inhibition caused ER stress and significantly reduced cell tumorigenicity, which was related to a significant downregulation of glucose-regulated protein 78 (GRP78) (4). GRP78, a modulator of UPR, is an antiapoptotic protein, which is widely upregulated in cancer and serves a key role in chemotherapy resistance in several types of cancer (22).

RRBP1 promotes the ribosome-independent localization of a subset of mRNA to the ER (23). p180 is required for efficient ER-anchoring of bulk poly(A) and certain transcripts, such as placental alkaline phosphatase and calreticulin, to the ER (23). The present study indicated that RRBP1 knockdown increased CCR7 mRNA and suppressed CCR7 protein expression compared with the control group, which might be due to RRBP1 knockdown restricting the combination between CCR7 mRNA and ER. Therefore, further research should be performed.

The present study had several limitations. Firstly, the clinicopathological characteristics of RRBP1 were analyzed using TCGA data. Therefore, the association between RRBP1 expression and the clinicopathological characteristics of patients requires further investigation. Secondly, multivariate analysis should be performed to confirm whether high RRBP1 expression is an independent prognostic factor in the clinical data. Thirdly, a rescue experiment was not conducted to validate the role of CCR7. Fourthly, the lack of additional cell lines in the functional experiments was also a limitation.

In conclusion, the present study indicated that RRBP1 was associated with bladder cancer cell migration, invasion and prognosis. RRBP1 may serve as a key player in the maintenance of tumor tumorigenesis, and CCR7 might serve a role in the process. The role of RRBP1 in bladder cancer tumorigenesis requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HP and NX analyzed the data. MW contributed to writing the manuscript and performing the statistical analyses. MW, SL and BZ performed the experiments and collected the data. MW and JW analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Beijing Chaoyang Hospital's Institutional Research Ethics Board (approval no. 2020-3-18-85). All subjects provided written informed consent after oral consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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