CCNB1 is involved in bladder cancer pathogenesis and silencing CCNB1 decelerates tumor growth and improves prognosis of bladder cancer

XUE-XUAN WANG[1,2*], HUA-YU WU[3*], YING YANG[1], MIAO-MIAO MA[1], YI-WEI ZHANG[1], HAI-ZHEN HUANG[1], SHENG-HUA LI[4], SHANG-LING PAN[1,2], JUN TANG[1,2] and JUN-HUA PENG[1,2]

[1]Department of Pathophysiology, Guangxi Medical University; [2]Key Laboratory of Longevity and Aging-Related Diseases, Guangxi Medical University, Ministry of Education, Nanning, Guangxi 530021; [3]Medical Experimental Center, The First People's Hospital of Nanning, The Fifth Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530022; [4]Department of Urology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

Received February 14, 2023; Accepted June 8, 2023

DOI: 10.3892/etm.2023.12081

Abstract. In search of an effective therapeutic target for bladder urothelial carcinoma (BLCA), the present study aimed to investigate the expression of cyclin B1 (CCNB1) and its putative mechanism in BLCA. BLCA sequencing data from Gene Expression Omnibus and The Cancer Genome Atlas were used to analyze expression of CCNB1 mRNA and high CCNB1 expression had a poorer prognosis compared with those with low expression. Immunohistochemistry (IHC) samples collected from the Human Protein Atlas database were analyzed for CCNB1 protein expression. Short hairpin (sh) CCNB1-transfected BLCA T24 and 5637 cells were used to investigate the effects of CCNB1 and inhibit the proliferation, migration and invasion of BLCA cells, affect the cell cycle distribution and promote apoptosis of 5637 cells. A sh-CCNB1 BLCA chicken embryo chorioallantoic membrane (CAM) transplantation model was established to observe the impacts of sh-CCNB1 on the tumorigenesis of BLCA *in vivo*. Analysis of sequencing data showed that CCNB1 mRNA was significantly elevated in tumor and BLCA compared with normal tissues [standardized mean difference (SMD)=1.21; 95% CI: 0.26-2.15; I^2=95.9%]. IHC indicated that CCNB1 protein was localized in the nucleus and cytoplasm and was significantly increased in BLCA tumor tissues. The *in vitro* tests demonstrated that proliferation of T24 and 5637 cells transfected with sh-CCNB1 was significantly inhibited and cell migration and invasion ability were significantly decreased. sh-CCNB1 decreased the percentage of T24 cells in G0/G1, 5637 cells in the G0/G1 phase and S phase and increased percentage of 5637 cells in the G2/M phase and increased early apoptosis of 5637 cells. The *in vivo* experiments demonstrated that the mass of transplanted tumors was significantly decreased compared with the control group following silencing of CCNB1. The present results suggested that CCNB1 was involve in the development and prognosis of BLCA and silencing of CCNB1 may be a promising targeted therapy for BLCA.

Correspondence to: Professor Jun-Hua Peng or Professor Jun Tang, Department of Pathophysiology, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, P.R. China
E-mail: pengjh@gxmu.edu.cn
E-mail: tangjun@gxmu.edu.cn

*Contributed equally

Abbreviations: BLCA, bladder urothelial carcinoma; CAM, chorioallantoic membrane; HPA, Human Protein Atlas; KM, Kaplan-Meier

Key words: BLCA, cyclin B1, gene expression, cell proliferation

Introduction

Among urological malignancies, bladder urothelial carcinoma (BLCA) is the most common type of all urological malignancy, including those not caused by transitional epithelial tissue (1). Currently, surgery is the initial treatment option for BLCA, followed by chemotherapy with gemcitabine and other drugs (2). However, despite systemic therapy, BLCA still has high recurrence and progression rates and poor prognosis in China as at 2019, especially for those with muscular infiltration (3). Although numerous studies (4) have shown that tumor suppressor genes and proto-oncogenes serve key roles in the development of BLCA, to the best of our knowledge, their underlying mechanisms have not been clarified. Therefore, it is key to explore novel molecular markers and mechanisms of BLCA to improve its early diagnosis and prognosis.

Cyclin B1 (CCNB1), a member of the cyclin family located at locus 12 on the long arm of chromosome 5, is a key initiator of mitosis that can promote cell cycle progression (5,6) and exhibits changes in expression throughout the cell cycle. Several studies have confirmed that CCNB1 is highly expressed in numerous types of human tumor tissue and is related to tumor cell proliferation, metastasis and poor prognosis (7). Zhang *et al* (8) demonstrated that the CCNB1 expression levels and apoptosis in pancreatic cancer tumor

tissue are increased and the cell proliferation is decreased following silencing of CCNB1 via short hairpin (sh)CCNB1. Another report indicated that sh-CCNB1 arrests colorectal cancer cells in the G2/M phase and thereby inhibits proliferation (9). Zou *et al* (10) noted that the CCNB1 expression is positively correlated with infiltration levels of various inflammatory cells, including $CD4^+$ and $CD8^+$ T cells and negatively associated with prognosis of hepatocellular carcinoma and may serve as a potential prognostic biomarker for this liver malignancy. In another study, the mitosis-promoting factor CCNB1/CDK1 complex was found to regulate mitochondrial metabolism and ATP production; this might be an effective antineoplastic resistance-associated therapeutic target (11). Together, the aforementioned studies indicated that CCNB1 is highly expressed in multiple types of malignancy, including pancreatic, colorectal and liver cancer, and affects cell cycle regulation, proliferation and ATP synthesis of cancer cells and thus the development and prognosis of tumors. Several research groups have investigated the putative effects of CCNB1 in BLCA, but they mainly focused on the roles of CCNB1-related genes (12,13) and the roles and underlying mechanisms of CCNB1 in the development of BLCA remain unexplored.

The present study aimed to investigate the effects and possible mechanisms of CCNB1 in BLCA using public databases, followed by primary confirmative experiments *in vitro* and *in vivo*, such as cell cycle and proliferation analysis, to determine its biological functions in BLCA cell models and the effect of CCNB1 on prognosis of patients with BLCA to find practical biomarkers for early diagnosis and intervention in BLCA.

Materials and methods

Materials. Normal bladder cells (SV-HUC-1) and human BLCA cells (T24, 5637 and UM-UC-3) were obtained from Shanghai Cell Bank (Chinese Academy of Sciences). RPMI-1640 and fetal bovine serum were obtained from Gibco (Thermo Fisher Scientific, Inc.). Penicillin-streptomycin double antibody was obtained from Beijing Solarbio Science & Technology Co., Ltd. Trypsin and CCNB1 and GAPDH primers were obtained from Sangon Biotech Co., Ltd. RNA extraction (Axygen) and Transwell chambers were obtained from Corning, Inc. HiScript® III RT SuperMix for qualitive (q)PCR and Cell Counting Kit (CCK)-8 were obtained from Vazyme Biotech Co., Ltd. RIPA lysis solution and BCA quantification kit were obtained from Beyotime Institute of Biotechnology. Chickens embryos (age, 7 days) were obtained from Guangxi Fufeng Agricultural and Livestock Company.

Bioinformatics analysis. CCNB1 mRNA expression in pan-cancer was obtained from The Cancer Genome Atlas (TCGA; https://www.cancer.gov/ccg/research/genome-sequencing/tcga) database. CCNB1 mRNA expression data were obtained for both BLCA and non-cancerous tissue, including 144 BLCA cases and 19 non-cancerous controls. BLCA-associated high-throughput sequencing data were obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo) database. A total of 20 datasets from 10 platforms were utilized (accession nos. GPL96, GPL570, GPL6102, GPL14951, GSE19915, GSE24152, GSE40355, GSE52519 and GSE76211), including 742 BLCA and 173 non-cancerous samples (Fig. S1; Table SI). The expression matrix was transformed by uniform Log2 calculation and the batch effect was removed using the removeBatchEffect function in R version 4.2.1 Limma package to normalize data. The summary standardised mean differences (SMD) were estimated using meta-analysis with random effects. Kaplan-Meier univariate survival analysis (KM survival curve) of the ENCORI (http://starbase.sysu.edu.cn/index.php) online database. The protein expression of CCNB1 in BLCA and normal bladder tissue was investigated in randomly chosen clinical samples (Patient ID: 2053, 2311, 2031 and 2699, n=4) from the Human Protein Atlas (HPA) database (proteinatlas.org/).

Cell culture and transfection. T24 and 5637 BLCA cells were cultured at 37˚C and 5% CO_2 in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin double antibody. The cultured T24 and 5637 cells were randomly divided into experimental group and the negative control (NC) group. The viral solution obtained following lentiviral packaging of sh-CCNB1 (5'-gcCAAATACCTGATGGAACTA-3') and sh-NC (5'-TTCTCCGAACGTGTCACGT-3') plasmid with the HIV backbone-based lentiviral particle packaging kit and empty viral backbone (Lenti-Pac™ HIV Expression Packaging Kit; GeneCopoeia, Inc.) were used to transfect T24 and 5637 cells. The lentiviral backbone was pCMV-hU6-MCS-Ubiquitin-EGFP-IRES-puromycin (Shanghai GeneChem Co., Ltd.). Opti-MEM serum-free medium (200 μl) was added with 2.5 μg of plasmid and 5 μl of Lenti-Pac and marked as tube A. An additional 200 μl opti-MEM was used in the absence of serum medium, a total of 15 μl of EndoFectin Lenti was added, and marked as tube B. Tubes A and B were mixed to transfect 293T cells until 4 days in order to collect lentivirus. Temperature of transfection for T24 and 5637 cells was 37˚C and lasted for 48 h. After screening with 2 μg/ml puromycin for at least 3 days before additional experiments were performed, the proportion of cells with green fluorescent protein was observed under a fluorescent microscope (magnification, x100). Subsequent experiments were performed when the percentage of cells with green fluorescent protein was >70% of the total number of cells.

Detection of CCNB1 mRNA expression in BLCA cells by reverse transcription-quantitative (RT-q)PCR. Total RNA was harvested from cells (Axygen Scientific Inc) and reverse transcriptase was used to synthesize cDNA (HiScript III RT SuperMix for qPCR). The system of comprised 4 μl 4X gDNA wiper mix, 1 μl RNA and 11 μl RNase-free water to 42˚C for 2 min. 4 μl 5X HiScript III q-RT SuperMix was added at 37˚C for 15 min, 85˚C for 5 sec and stored at 4˚C. The cDNA was diluted three times for RT-qPCR reaction. The reaction system comprised 10 μl 2X FS Universal SYBR Green Master Mix (FS Universal SYBR Green Master; Roche Diagnostics), 0.6 μl each of the upstream and downstream primers, 2 μl cDNA and RNase-free water to a total volume of 20 μl. Thermocycling conditions were as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 10 sec, 65˚C for 1 min and 97˚C for 1 sec and final extension at 37˚C for 30 sec. The primer sequences of CCNB1 and the internal reference control

GAPDH were amplified separately. The primer sequences were as follows: CCNB1 forward, 5'-GCCTGAGCCTATTTT GGTTGATAC-3' and reverse, 5'-TCCATCTTCTGCATCCAC ATCA-3' and GAPDH (internal control) forward, 5'-ACCACA GTCCATGCCATCAC-3' and reverse, 5'-TTCCCGTTCAGC TCAGGGAT-3'. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression ($2^{-\Delta\Delta Cq}$ Livak and Schmittgen 2001).

CCK-8 assay. When the density of the stable cell lines at the logarithmic growth stage reached 70-80%, the cells were digested with trypsin and counted by counting board. Cell suspension (100 μl containing 2,500 cells/well) was added to a 96-well plate with triplicate wells. After 1 h incubation with 10 μl CCK-8 solution, the cells were assessed at 490 nm using an enzyme marker to assess the cells at 490 nm and the measured OD value indirectly reflects the number of viable cells. A cell proliferation curve was drawn.

Transwell assay for cell migration and invasion. Transwell chambers were sterilized by radiation under UV light for 2 h at room temperature. The RPMI-1640 medium containing 5% fetal bovine serum was added to each well of the 24-well plate and Transwell chambers were gently placed into the wells to avoid air bubbles. Then, the upper chamber was filled with a cell suspension containing $6x10^4$ cells in a volume of 100 μl. It was incubated for 24 h at the incubation temperature of 37˚C. The cells were gently wiped from the chamber with a cotton swab. Following washing with PBS solution three times, 500 μl 70% methanol solution was added, fixed at 37˚C for 30 min, and put into a 3% crystal violet solution for overnight staining at room temperature. Chambers were dried with a clean cotton swab and images captured under a microscope. The quantity of cells that had penetrated the membrane was recorded. For cell invasion assay, the ratio of the Matrigel matrix gel at 4˚C to the serum-free RPMI-1640 medium was 1:8, and 60 μl solution was spread evenly on the upper chamber for 30 min. Medium plated in upper chamber (usually without serum) and medium and serum (type and concentration) plated in lower chamber. The remaining procedures were the same as the migration assay. Observation was with a light microscope (magnification, x100). A total of three independent repeats was performed.

Cell cycle and apoptosis analysis. When the cell density in the culture dish was ~70%, the RPMI-1640 medium containing 10% fetal bovine serum was replaced by serum-free RPMI-1640 medium. After 12 h at 37˚C, the 10% fetal bovine serum of RPMI-1640 medium was substituted by the serum-free RPMI-1640 medium to synchronize the cell cycle. After another 12 h at 37˚C, $\geq 1x10^6$ cells were extracted following transfection, washed twice with pre-cooled PBS solution and centrifuged at 300 x g for 5 min at 37˚C. A total of 1 ml PBS solution was added and the sample was resuspended after supernatant was discarded. The cells were homogenized by adding 1 ml PBS solution and shaken on a vortex shaker while adding 3 ml 75% isopropyl alcohol dropwise. The solution was fixed by 1 ml PBS solution and 3 ml 75% isopropyl alcohol overnight at -20˚C in the dark. It was centrifuged at 300 x g for 5 min at 37˚C. The supernatant was discarded and cell precipitation were cleaned with 3 ml PBS. Then, 1 ml PI/RNase dye was added for cell cycle analysis by flow cytometry (BD FACSCalibur; BD Biosciences). The cells were shielded from light for 30 min before being tested. For apoptosis detection, the cells were centrifuged at 300 x g at 37˚C for 5 min and resuspended in 500 μl 1X binding buffer. A total of 5 μl Annexin V-FITC and 10 μl PI dye were added to each sample, incubated for 15 min with gentle shaking and then incubated at room temperature for 1 h before apoptotic analysis by flow cytometry (BD FACSCalibur; BD Biosciences). FlowJo 7.6 (FlowJo LLC) was used for analysis.

Tumorigenic experiment on chick chorioallantoic membrane (CAM). The air chambers of CAM from 7-day-old chicken embryos at 42˚C were outlined by a marker after checking for signs of life: In the dark room, blood vessels and embryonic movements were clearly visible under a flashlight. The tumorigenic experiment was performed as follows. The eggshell was knocked on at the air chamber of the circle and the air chamber membrane soaked with 0.9% normal saline until blood vessels could be clearly observed. Tweezers were used to tear the air chamber membrane where the blood vessels were rare and exposed the CAM. Finally, a silicon ring (diameter 7 mm) was placed between two large blood vessels.

BLCA cells ($1.2x10^7$) at the logarithmic growth stage were collected and added into the silicon ring, which was closed with sterile and breathable adhesive tape. The signs of life, tumor size and the surrounding blood vessels of embryos were observed with a torch every 24 h. At 6 days after cell implantation, intact tumors were removed and measured, then stored in 4% paraformaldehyde at room temperature for three days. The sections were cut at 4 μm and stained with hematoxylin solution for 3-5 min, washed with 85% ethanol for 5 min, 95% ethanol for 5 min and stained with eosin for 5 min. The sections were observed with a light microscope (magnification, x400).

Statistical analysis. Data were analyzed using SPSS 25.0 (IBM Corp.) and images were drawn using GraphPad Prism 8. Data (Dotmatics) were tested for normality. Data adhering to a normal distribution are presented as the mean ± SD. Independent unpaired t test was used for comparisons between two groups; the Analysis of Variance (ANOVA) was performed for comparisons between >2 groups. For measures that did not have a normal distribution, descriptive statistics are reported as the median and interquartile range. Non-parametric tests were used to compare groups and correlation was analyzed using Spearman's correlation coefficient. The real standardized mean difference (SMD) was calculated using Stata 15.0 (StataCorp LLC) and forest plots and the summary receiver operating characteristic (sROC) curves were plotted. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CCNB1 expression is increased in BLCA. The expression of CCNB1 mRNA in numerous types of cancers and non-cancer tissues of TCGA database were integrated for analysis. Numerous types of tumor tissues had notably greater levels of CCNB1 mRNA than normal tissue, except for certain tumors such as thymic carcinoma (Fig. 1A). In the online

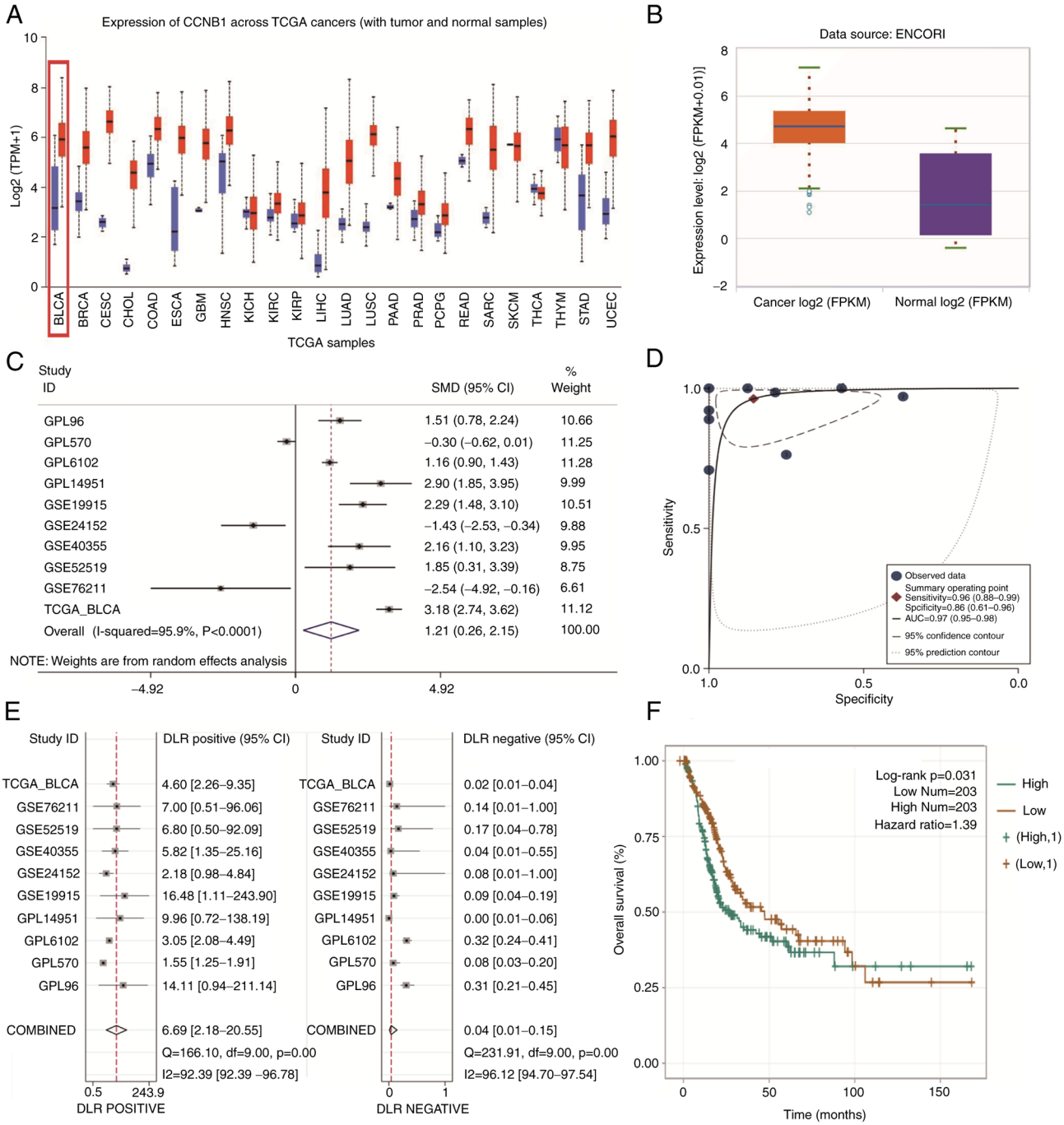

Figure 1. CCNB1 mRNA expression in pan-cancer and BLCA. (A) CCNB1 was expressed at notably higher levels in pan-cancer than in normal tissue. (B) Expression of CCNB1 was notably higher in BLCA than in normal bladder tissue in the online database ENCORI (P<0.05). (C) Forest plots of eligible datasets in TCGA and GEO databases showed high expression of CCNB1 in BLCA tissue. (D) sROC AUC suggested good diagnostic value of CCNB1. (E) Combined positive likelihood ratio was 6.69 (2.18-20.55) and the combined negative likelihood ratio was 0.04 (0.01-0.15) (P<0.05). (F) Kaplan-Meier survival curve suggested that patients with BLCA and high CCNB1 expression had worse prognosis than those with low expression (P<0.05). CCNB1, ; BLCA, ; TCGA, ; GEO, ; sROC, ; AUC, ; KM, ; FPKM, ; TPM, ; DLR,.

database TCGA, 411 BLCA samples and 19 normal bladder tissue samples were analyzed. CCNB1 mRNA expression was notably higher in BLCA than in normal bladder tissue (Fig. 1B).

Meta-analysis of the GEO microarray data and TCGA database data showed that CCNB1 mRNA expression levels were markedly higher in BLCA than in non-cancerous bladder tissue (SMD=1.21; 95% CI, 0.26-2.15; I^2=95.9%; Fig. 1C); the area under the sROC curve was 0.97 (95% CI: 0.95-0.98; Fig. 1D). Stata 15.0 yielded a combined positive likelihood ratio of 6.69 and a combined negative likelihood ratio of 0.04 for CCNB1 (Fig. 1E). These results suggested that the high expression of CCNB1 mRNA distinguished BLCA from normal bladder tissue samples. Kaplan-Meier univariate survival analysis of the ENCORI online database demonstrated that patients with BLCA and high CCNB1 expression had a significantly decreased overall survival time and poorer prognosis than those with low expression (Fig. 1F).

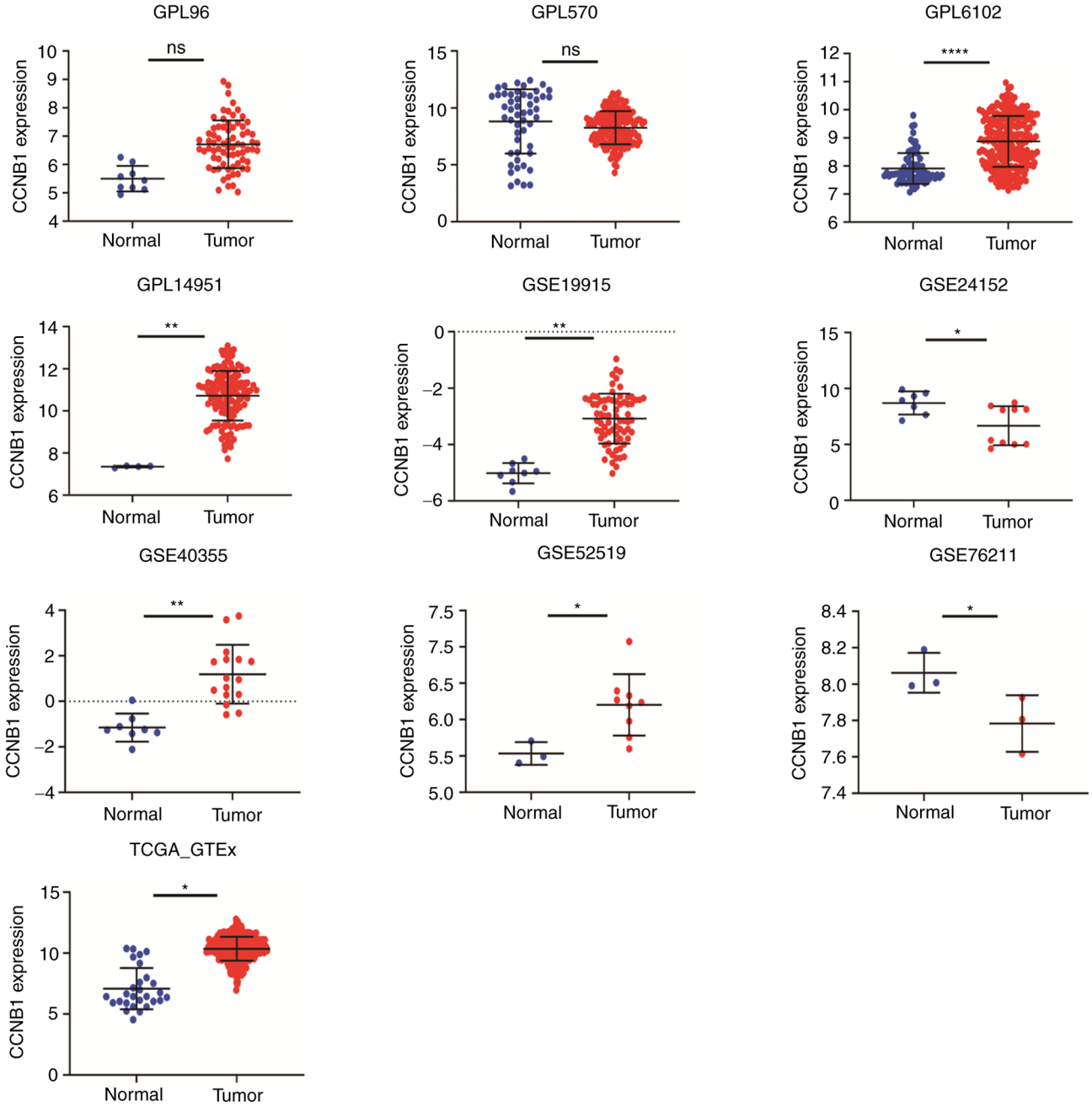

Figure 2. Expression of CCNB1 in datasets. A total of six datasets showed significantly high expression, including GPL6102, GPL14951, GSE19915, GSE40355, GSE52519 and TCGA-GTEx. *P<0.05, **P<0.01, ****P<0.0001. CCNB1, cyclin B1; TCGA, The Cancer Genome Atlas; ns, not significant.

CCNB1 expression differed between BLCA and non-BLCA bladder tissues in 11 datasets and showed significantly higher expression in six datasets, including GPL6102, GPL14951, GSE19915, GSE40355, GSE52519 and TCGA-GTEx (Fig. 2).

To validate the protein expression levels of CCNB1 in BLCA and normal bladder tissue, two groups of samples from the HPA database were randomly selected for analysis. Protein levels were substantially increased in BLCA compared with normal bladder tissue (Fig. 3).

Expression of CCNB1 mRNA in normal and bladder cancer cells. Expression of CCNB1 mRNA in normal bladder cells (SV-HUC-1) and human BLCA cells (T24, 5637 and UM-UC-3) was detected by RT-qPCR. The relative expression of CCNB1 mRNA in bladder cancer cells was higher than in normal bladder cells and the relative expression of CCNB1 mRNA in T24 and 5637 cell lines was compared with UM-UC-3 cells (Fig. 4). Therefore, T24 and 5637 cell lines were selected for silencing CCNB1.

Establishment of cell lines with stable expression of sh-CCNB1. T24 and 5637 cell lines were infected with the virus solution obtained after lentiviral packaging of sh-CCNB1 plasmid and sh-CCNB1 stably expressed cell lines were established after puromycin selection. Silencing efficiency of CCNB1 was measured by RT-qPCR, yielding 75 and 71%, respectively (Fig. 5A and B). Western blotting and RT-qPCR showed that the expression levels of CCNB1 protein and mRNA were

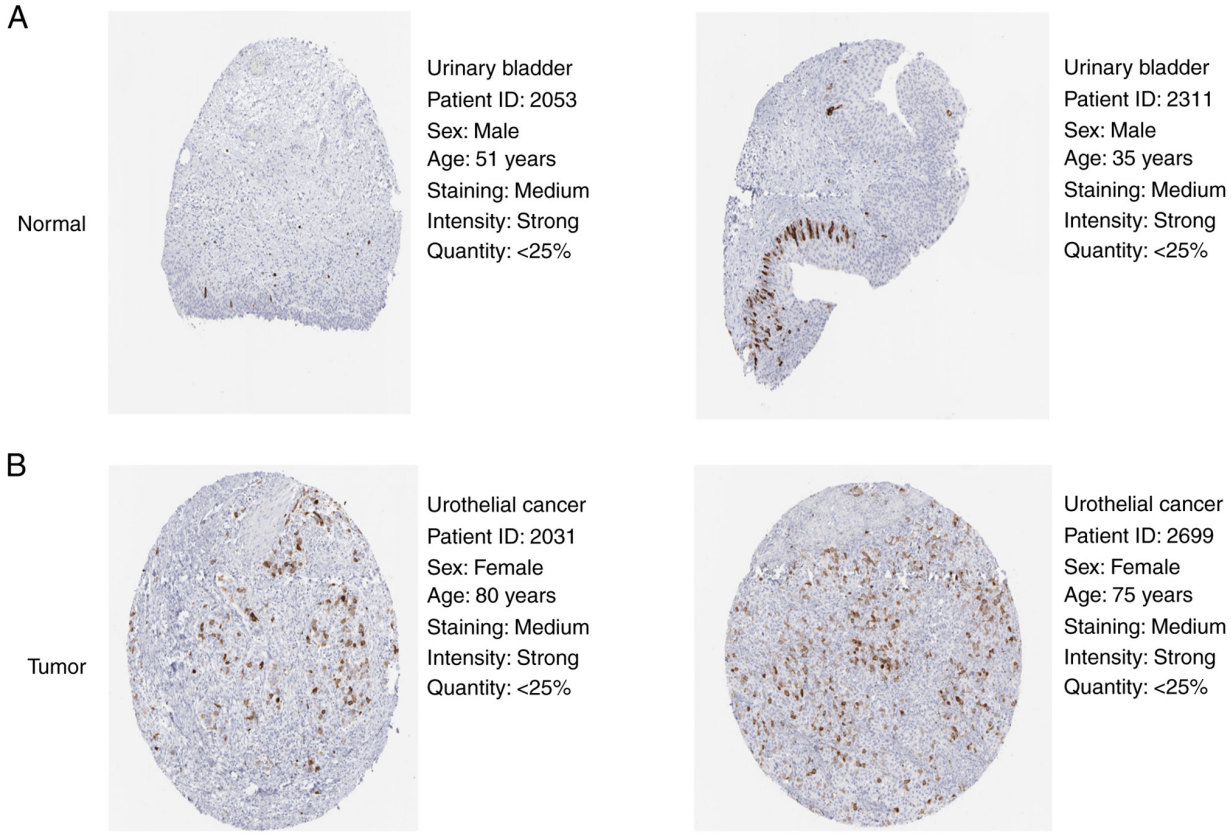

Figure 3. Immunohistochemical staining and clinical information of normal bladder and BLCA tissues in Human Protein Atlas. Expression of cyclin B1 protein in (A) normal bladder and (B) BLCA tissue. BLCA, bladder urothelial carcinoma.

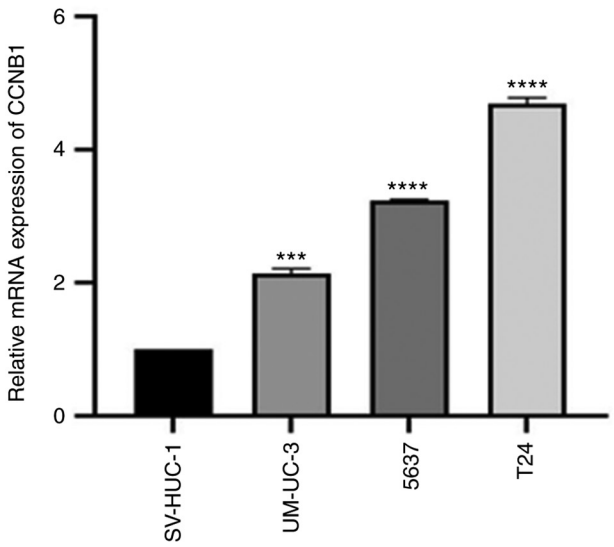

Figure 4. Expression of CCNB1 mRNA in normal and bladder cancer cell lines. ***P<0.001, ****P<0.0001 vs. UM-UC-3 cells. CCNB1, cyclin B1.

significantly decreased following sh-CCNB1 transfection compared with sh-NC, indicating that stable expression of sh-CCNB1 was in T24 and 5637 cells.

Inhibition of CCNB1 expression decreases the migration and invasion of BLCA cells. The ability of cells to migrate and invade was examined by Transwell assay. Compared with sh-NC group, sh-CCNB1 group in both cell lines showed a significant reduction in cell migration and invasion (P<0.0001 and P<0.001, respectively; Fig. 5C and D).

sh-CCNB1 inhibits the proliferation of BLCA cells, affects the cell cycle distribution and promotes apoptosis of 5637 cells. CCK-8 showed that compared with sh-NC group, sh-CCNB1 group of T24 and 5637 cells exhibited significantly lower proliferative capacity at day 6 (P<0.0001; Fig. 6A). The effect of sh-CCNB1 on BLCA cell cycle and apoptosis was observed by flow cytometry, with sh-CCNB1 decreasing the percentage of T24 cells in G0/G1 (P<0.05) 5637 cells in the G0/G1 phase (P<0.0001) and S phase (P<0.01) and increasing percentage of 5637 cells in the G2/M phase (P<0.0001; Fig. 6B). sh-CCNB1 induced early apoptosis in 5637 cells (P<0.05; Fig. 6C), but had no significant effect on the apoptosis of T24 cells.

sh-CCNB1 inhibits tumorigenesis in CAM. T24 and 5637 cells were inoculated into the CAM and the survival of the chicken embryo was observed every 24 h. The tumor cell suspension floated on the surface of the CAM in a thin film within 24 h of inoculation, with no notable change in the blood vessels. At day 6, the tumor protruded and small blood vessels were observed in surrounding area. By day 6, the tumor appeared in the AM, protruding from the CAM, which presented clear borders. When small blood vessels were seen observed in a radial pattern around the tumor center, the growth of chicken embryos were terminated and tumors were removed at sixth day after inoculation. Volumes of T24-sh-CCNB1

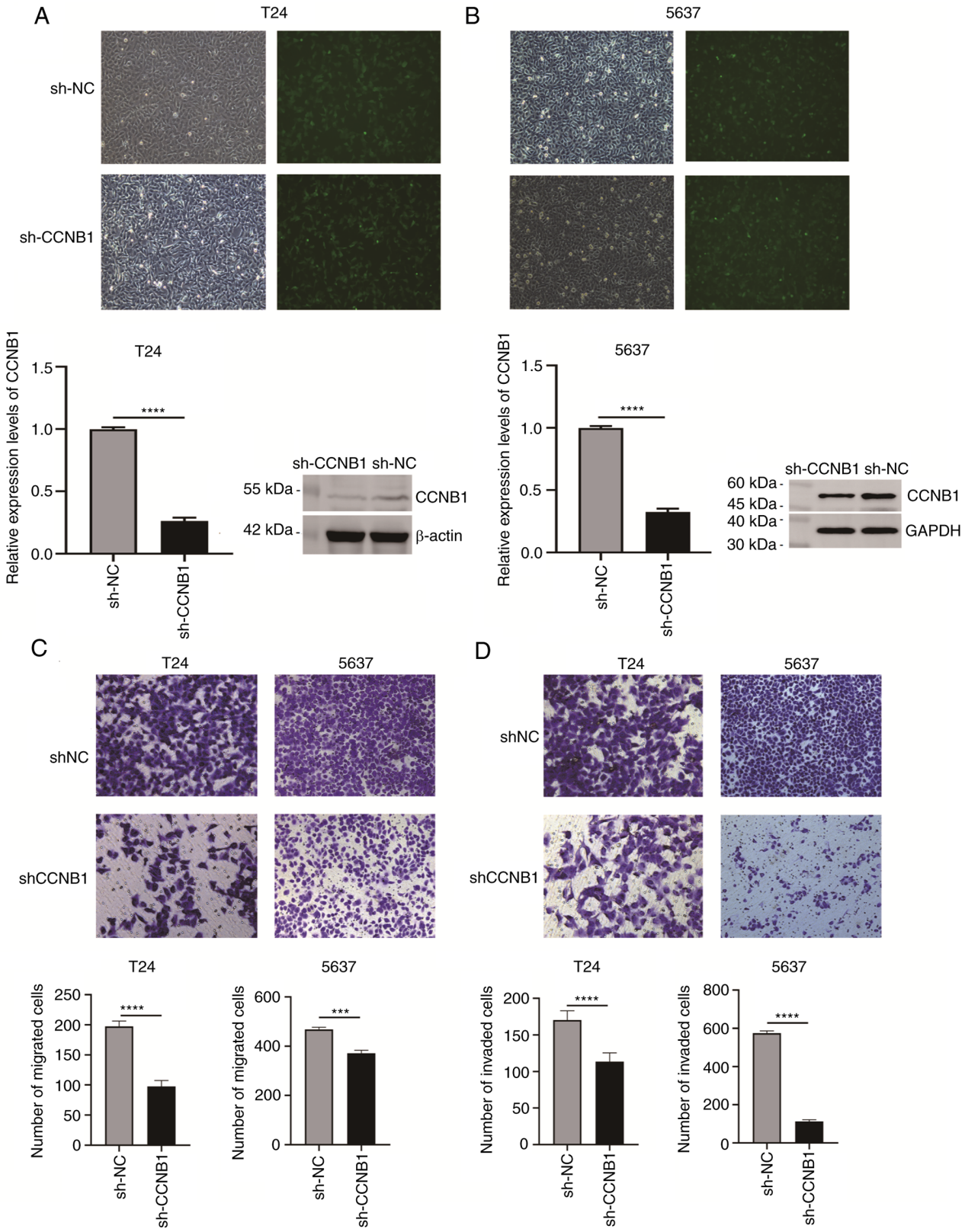

Figure 5. Construction of CCNB1-silenced stably transfected cell lines and migration and invasion of bladder cancer cell lines. (A) Representative fluorescence microscopy of (A) T24 and (B) 5637 cells infected with sh-NC and sh-CCNB1. Reverse transcription-quantitative PCR detection of silencing efficiency was 75 and 71%, respectively. CCNB1 protein expression was significantly decreased. sh-CCNB1 inhibited (C) migration and (D) invasion of T24 and 5637 cells. Magnification, x100. ***P<0.001, ****P<0.0001. CCNB1, cyclin B1; sh, short hairpin; NC, negative control.

and 5637-sh-CCNB1 derived tumors were smaller compared with sh-NC group (Fig. 7A). The diameter of the implanted tumor was measured and its volume was calculated after it was separated from CAM (Table I). This experiment was repeated three times and it was concluded that the volumes of T24-sh-CCNB1 and 5637-sh-CCNB1 were smaller than

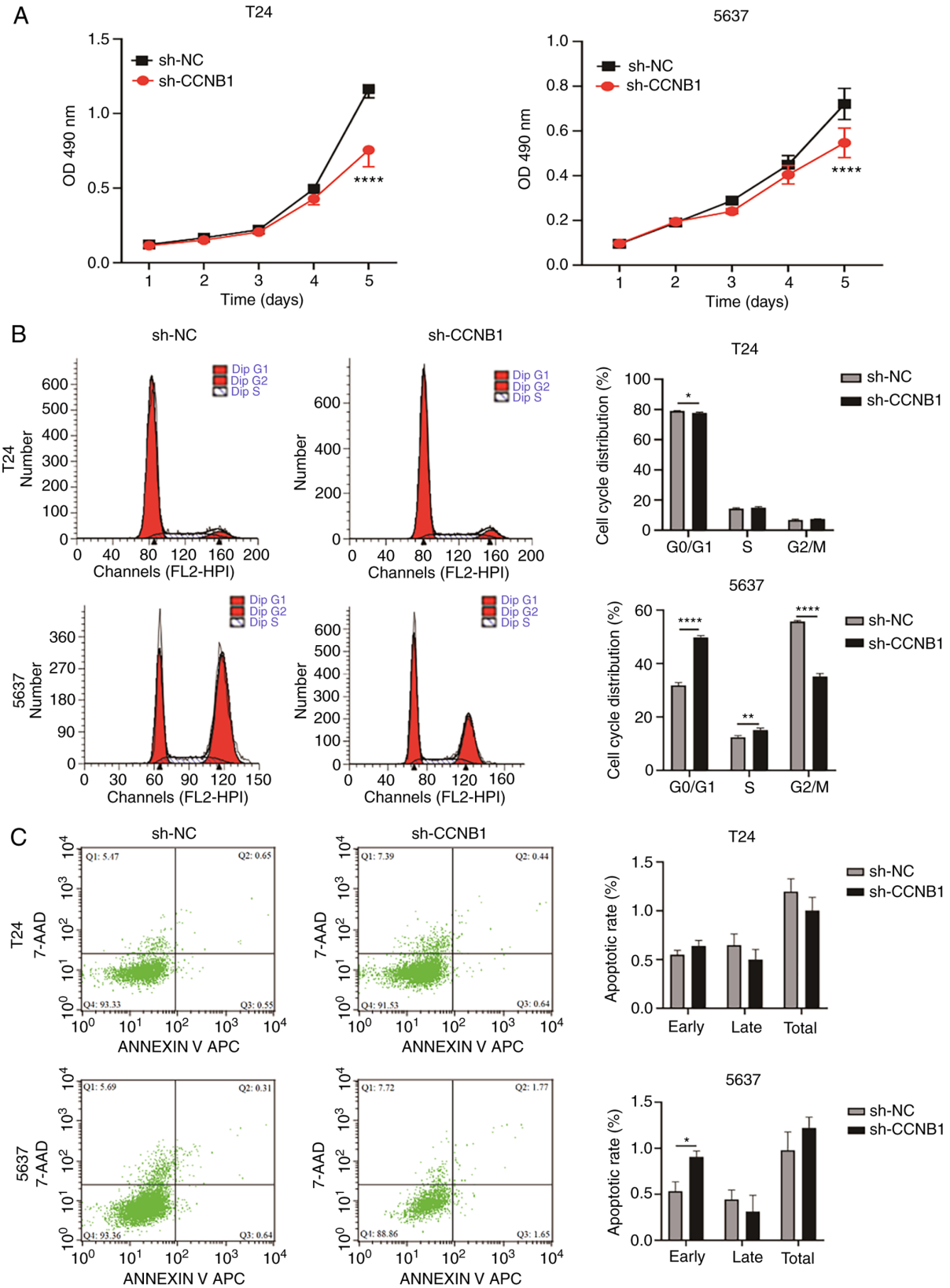

Figure 6. Effect of sh-CCNB1 on proliferation, cell cycle and apoptosis of T24 and 5637 cells. (A) sh-CCNB1 significantly inhibited cell proliferation. (B) sh-CCNB1 decreased percentage of T24 and 5637 cells in G0/G1 and S and increased percentage in G2/M phase. (C) In 5637 cells, sh-CCNB1 induced early apoptosis. *P<0.05, **P<0.001, ****P<0.0001. sh, short hairpin; CCNB1, cyclin B1; NC, negative control; OD, optical density.

the sh-NC group. Under light microscope in the group of T24-sh-CCNB1 and 5637-sh-CCNB1, HE-stained invasive cancer cells were morphologically changed, exhibiting enlarged, angular or irregular, dark-stained nuclei, some of which presented small or double nucleoli. Fibrous connective tissue reaction was observed in the interstitial tissue, with

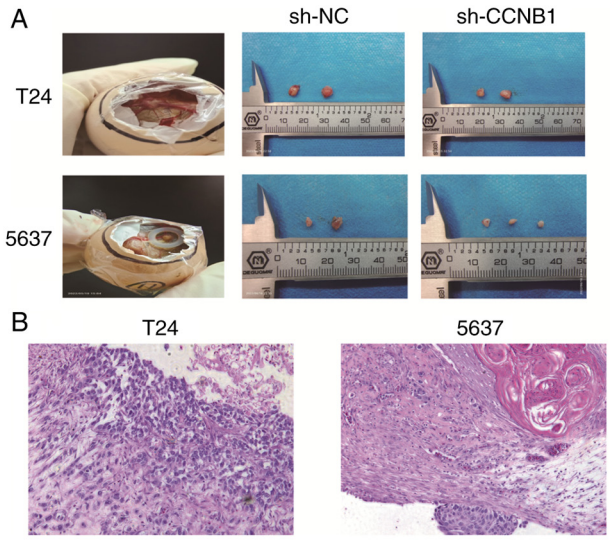

Figure 7. Transplanted tumors in CAM and pathological alterations of transplanted tumors. (A) Transplanted tumors of T24 and 5637 cells transfected with sh-CCNB1. (B) Hematoxylin-eosin-stained invasive cancer cells were morphologically changed (magnification, 400x). CAM, chorioallantoic membrane; sh, short hairpin; CCNB1, cyclin B1; NC, negative control.

different degree of plasma cell and lymphocyte infiltration. Squamous and glandular differentiation was observed in focal cancer cells (Fig. 7B).

Discussion

To date, curing cancer remains a challenge for humans (14). The application of cell cycle protein family in tumor therapy provides a novel direction for cancer treatment (15). Many studies have demonstrated that CCNB1 serves critical roles in tumor progression (16), but, to the best of our knowledge, its effects and mechanisms in BLCA have not yet been reported. To the best of our knowledge, the present study is the first to demonstrate that CCNB1 was highly expressed in BLCA. sh-CCNB1 was found to inhibit the proliferation, invasion, migration and tumor growth of BLCA cells, indicating key roles in cell functions and tumorigenesis of BLCA. Additionally, there was an association between high CCNB1 expression and poor prognosis for patients with BLCA. Therefore, exploring the precise roles of CCNB1 in BLCA may provide a theoretical foundation for future clinical treatment of BLCA.

As a member of the cell cycle protein family, CCNB1 is highly expressed in various types of tumor tissues and is involved in tumorigenesis by regulating cell proliferation and cycle distribution. For example, CCNB1 mRNA is highly expressed in gastric cancer and serves as a downstream target gene of heterogeneous nuclear ribonucleoprotein R (hnRNPR) protein, to which it binds to form the hnRNPR/CCNB1/Centromere Protein F (CENPF) mRNA axis, thus promoting proliferation of gastric cancer cells (17); another study found that CCNB1 partially reverses increased proliferation of gastric cancer cells (18). In an ovarian cancer study, high expression of CCNB1 is negatively regulated by microRNA-559, thus promoting proliferation of ovarian cancer cells *in vitro* and increasing the lung metastasis of ovarian cancer cells *in vivo* (19). By contrast, silencing of

Table I. Bladder urothelial carcinoma cell transplant tumor volume after 6 days.

Group	Mean diameter, mm	Mean volume, mm^3
T24-sh-NC	5.00	22.50
T24-sh-CCNB1	2.75	12.38[a]
5637-sh-NC	3.25	14.63
5637-sh-CCNB1	1.33	5.985[a]

Tumor volume was calculated as follows: Length x $width^2$ x0.5. Comparison between groups of homogeneous cells, [a]P<0.05. sh, short hairpin; NC, negative control; CCNB1, cyclin B1.

CCNB1 inhibits tumor cell proliferation (20). Taken together, increasing evidence suggests that a high expression of CCNB1 promotes cancer cell proliferation and tumorigenesis (21). It was hypothesized that CCNB1 may similarly impact the pathogenesis of BLCA. Therefore, the present study constructed a BLCA and CAM model with sh-CCNB1. As expected, the proliferation of BLCA cells and tumor growth in CAM was significantly inhibited by sh-CCNB1.

As an allosteric modulator for cyclin-dependent kinases CDK1 and Cdc2, CCNB1 had been documented to inhibit tumor growth by influencing cell cycle events (22). For example, in renal cell carcinoma, the downregulation of CCNB1 and CDK1 expression causes G2/M arrest and apoptosis in tumor cells, indicating that upstream eukaryotic translation initiation factor 3 subunit D (EIF3D) is involved in development of renal cell carcinoma as a potential proto-oncogene (23). Another study demonstrated that diacetin, which is a symptomatic slow acting drug in osteoarthritis, inhibits proliferation of chondrosarcoma cells by inducing G2/M cell cycle arrest via downregulation of CDK1/CCNB1 (24). Similarly, CCNB1, as a key regulator of cell cycle and promoter of G2/M phase, improves the prognosis of platinum-based chemotherapy in advanced non-small cell lung cancer (25). To determine the mechanism underlying the effects of CCNB1 on BLCA, the present study analyzed the cell cycle distribution and apoptotic rate *in vitro*. Flow cytometry demonstrated that 5637 cells were significantly arrested in the G2/M phase, the proportion of G0/G1 phase was relatively decreased and the proportion of T24 cells in G0/G1 phase decreased. These findings demonstrated that sh-CCNB1 arrested BLCA cells at G2/M phase and inhibited BLCA cell proliferation.

Studies have investigated the potential roles of CCNB1 in BLCA (26), demonstrating that overexpressing G_2 and S phase-expressed 1 (GTSE1) promotes BLCA cell proliferation, invasion and migration through the P53/FoxM1/CCNB1 pathway and is positively associated with disease recurrence history, lymph node invasion and progression (27). Differential gene enrichment analysis by analyzing sequencing data from BLCA clinical samples shows that CCNB1 is a hub gene expressed at 4.795-fold higher levels in BLCA than in normal tissue, correlating with overall survival (28). However, the primary aim of the aforementioned studies was to explore the role of the genes associated with CCNB1, not CCNB1. To

the best of our knowledge, the present study was the first to construct *in vivo* and *in vitro* models to determine the role of CCNB1 in BLCA. CCNB1 mRNA expression and its clinical significance in patients with BLCA was assessed. Analysis of 1149 BLCA and 201 non-cancerous bladder tissue samples in database sequencing data confirmed high expression of CCNB1 in BLCA. Furthermore, the KM survival curve suggested that patients with BLCA with high levels of CCNB1 had a poorer prognosis than those with low levels of CCNB1, which indicated that CCNB1 may serve as a prognostic indicator for patients with BLCA. RT-qPCR, western blotting and *in vitro* cell function and *in vivo* CAM xenograft assay were performed to determine the effects of sh-CCNB1 on proliferation, invasion, migration, cell cycle progression and apoptosis of BLCA cells. The present results confirmed that sh-CCNB1 inhibited proliferation, invasion and migration of BLCA T24 and 5637 cells *in vitro*, significantly increased proportion of cells in G2/M phase and inhibited growth of xenograft tumors *in vivo*. Furthermore, the high expression of CCNB1 mRNA could distinguish BLCA from normal bladder tissue. CCNB1 in tumor cells as a cell cycle progression modulator may be an effective indicator to assess the malignancy of tumors (29). Therefore, targeting CCNB1 may be a promising option for BLCA therapy.

The present study had limitations. First, bigger clinical samples are required to validate the prognostic significance of CCNB1 in BLCA. Second, although experiments on chicken CAM transplantation tumors were performed, experimental sample size should be increased. *In vivo* experiments on other animals should also be conducted for validation, such as tumor formation in nude mice. CCNB1 protein levels were significantly higher in BLCA compared with normal bladder tissue from immunohistochemical results of clinical samples in the HPA database. This was not confirmed in clinical samples because it is difficult to obtain normal bladder tissue as controls. Finally, the present study only investigated the putative mechanism of action of CCNB1 in BLCA. Information on the molecular mechanism is lacking and the specific signaling pathways involved need further investigation.

In summary, the present study demonstrated that CCNB1 expression was upregulated in BLCA tumor tissue and sh-CCNB1 inhibited proliferation of BLCA cells, arresting most cells in the G2/M phase and thereby inducing apoptosis. The present study established a foundation for a understanding of CCNB1 function in BLCA. CCNB1 may have a key role in the progression of BLCA. Silencing of CCNB1 may have inhibited growth of BLCA tumors. The present study may provide theoretical foundation for future clinical treatment of BLCA.

Acknowledgements

The authors would like to thank Dr Fang-Cheng Jiang (Guangxi Medical University, Nanning, China) for assistance with bioinformatics analysis.

Funding

The present study was supported by Guangxi Natural Science Foundation (grant no. 2020GXNSFAA238027) and National Natural Science Foundation of China (grant nos. 81860205 and 32060188).

Availability of data and materials

The datasets presented in this study (including GSE2361, GSE3167, GSE5287, GSE2109, GSE7476, GSE17906, GSE31189, GSE31684, GSE13507, GSE19423, GSE37815, GSE37817, GSE65635, GSE86411, GSE19915, GSE24152, GSE40355, GSE52519, GSE76211, TCGA-GTEx) can be found in online databases: Gene Expression Omnibus (ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (genome.gov/).

Authors' contributions

JP and JT confirm the authenticity of all the raw data. XW performed all experiments, analyzed relevant data and drafted the manuscript. HW participated in the design of most experiments and proposed modifications. YY participated in the design of some of the experiments. MM participated in the analysis of the experimental data and revised the manuscript. YZ and HH participated in the collection and analysis of bioinformatics data. SL participated in the analysis of some of the experimental data and proposed various suggestions. SP, JT and JP conceived and designed the present study, provided financial support and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Xu Y, Wu G, Li J, Li J, Ruan N, Ma L, Han X, Wei Y, Li L, Zhang H, *et al*: Screening and identification of key biomarkers for bladder cancer: A study based on TCGA and GEO Data. Biomed Res Int 82: 83401, 2020.
2. Schlack K, Boegemann M, Steinestel J, Schrader A and Krabbe L: The safety and efficacy of gemcitabine for the treatment of bladder cancer. Expert Rev Anticancer Ther 16: 255-271, 2016.
3. Shi S and Tian B: Identification of biomarkers associated with progression and prognosis in bladder cancer via co-expression analysis. Cancer Biomark 24: 183-193, 2019.
4. Cao R, Yuan L, Ma B, Wang G, Qiu W and Tian Y: An EMT-related gene signature for the prognosis of human bladder cancer. J Cell Mol Med 24: 605-617, 2020.
5. Xie B, Wang S, Jiang N and Li JJ: Cyclin B1/CDK1-regulated mitochondrial bioenergetics in cell cycle progression and tumor resistance. Cancer Lett 443: 56-66, 2019.
6. Petrachkova T, Wortinger LA, Bard AJ, Singh J, Warga R and Kane D: Lack of Cyclin B1 in zebrafish causes lengthening of G2 and M phases. Dev Biol 451: 167-179, 2019.
7. Marques HP, da Silva SG, De Martin E, Agopian VG and Martins PN: Emerging biomarkers in HCC patients: Current status. Int J Surg 82s: 70-76, 2020.

8. Zhang H, Zhang X, Li X, Meng WB, Bai ZT, Rui SZ, Wang ZF, Zhou WC and Jin XD: Effect of CCNB1 silencing on cell cycle, senescence, and apoptosis through the p53 signaling pathway in pancreatic cancer. J Cell Physiol 234: 619-631, 2018.
9. Fang Y, Yu H, Liang X, Xu J and Cai X: Chk1-induced CCNB1 overexpression promotes cell proliferation and tumor growth in human colorectal cancer. Cancer Biol Ther 15: 1268-1279, 2014.
10. Zou Y, Ruan S, Jin L, Chen Z, Han H, Zhang Y, Jian Z, Lin Y, Shi N and Jin H: CDK1, CCNB1, and CCNB2 are prognostic biomarkers and correlated with immune infiltration in hepatocellular carcinoma. Med Sci Monit 26: e925289, 2020.
11. Hu L, Pan X, Hu J, Zeng H, Liu X, Jiang M and Jiang B: Proteasome inhibitors decrease paclitaxel-induced cell death in nasopharyngeal carcinoma with the accumulation of CDK1/cyclin B1. Int J Mol Med 48: 193, 2021.
12. Zeng S, Yu X, Ma C, Song R, Zhang Z, Zi X, Chen X, Wang Y, Yu Y, Zhao J, *et al*: Transcriptome sequencing identifies ANLN as a promising prognostic biomarker in bladder urothelial carcinoma. Sci Rep 7: 3151, 2017.
13. Wu F, Sun Y, Chen J, Li H, Yao K, Liu Y, Liu Q and Liu J: The oncogenic role of APC/C activator protein Cdc20 by an integrated pan-cancer analysis in human tumors. Front Oncol 11: 721797, 2021.
14. Dobruch J and Oszczudłowski M: Bladder cancer: Current challenges and future directions. Medicina (Kaunas) 57: 749, 2021.
15. Jamasbi E, Hamelian M, Hossain MA and Varmira K: The cell cycle, cancer development and therapy. Mol Biol Rep 49: 10875-10883, 2022.
16. Fang L, Du WW, Awan FM, Dong J and Yang BB: The circular RNA circ-Ccnb1 dissociates Ccnb1/Cdk1 complex suppressing cell invasion and tumorigenesis. Cancer Lett 459: 216-226, 2019.
17. Chen EB, Qin X, Peng K, Li Q, Tang C, Wei YC, Yu S, Gan L and Liu T: HnRNPR-CCNB1/CENPF axis contributes to gastric cancer proliferation and metastasis. Aging (Albany NY) 11: 7473-7491, 2019.
18. Li Y, Ji S, Fu LY, Jiang T, Wu D and Meng FD: Knockdown of cyclin-dependent kinase inhibitor 3 inhibits proliferation and invasion in human gastric cancer cells. Oncol Res 25: 721-731, 2017.
19. Yang X, Zhou S, Yang C, Cao C, He M and Zi S: CCNB1, negatively regulated by miR-559, promotes the proliferation, migration, and invasion of ovarian carcinoma cells. Mol Biotechnol 64: 958-969, 2022.
20. Gu J, Liu X, Li J and He Y: MicroRNA-144 inhibits cell proliferation, migration and invasion in human hepatocellular carcinoma by targeting CCNB1. Cancer Cell Int 19: 15, 2019.
21. Song Y, Zhao C, Dong L, Fu M, Xue L, Huang Z, Tong T, Zhou Z, Chen A, Yang Z, *et al*: Overexpression of cyclin B1 in human esophageal squamous cell carcinoma cells induces tumor cell invasive growth and metastasis. Carcinogenesis 29: 307-315, 2008.
22. Sun J, Du Y, Song Q, Nan J, Guan PZ, Guo JH, Wang X, Yang JB and Zhao CY: E2F is required for STAT3-mediated upregulation of cyclin B1 and Cdc2 expressions and contributes to G2-M phase transition. Acta Biochim Biophys Sin (Shanghai) 51: 313-322, 2019.
23. Pan XW, Chen L, Hong Y, Xu DF, Liu X, Li L, Huang Y, Cui LM, Gan SS, Yang QW, *et al*: Cui: EIF3D silencing suppresses renal cell carcinoma tumorigenesis via inducing G2/M arrest through downregulation of Cyclin B1/CDK1 signaling. Int J Oncol 48: 2580-2590, 2016.
24. Lohberger B, Leithner A, Stuendl N, Kaltenegger H, Kullich W and Steinecker-Frohnwieser B: Diacerein retards cell growth of chondrosarcoma cells at the G2/M cell cycle checkpoint via cyclin B1/CDK1 and CDK2 downregulation. BMC Cancer 15: 891, 2015.
25. Ying Y, Wang Z, Tan Y, Cao H, Gao H, Zhang Z, Zeng S and Xu C: Identification and validation of immunohistochemical marker panels to predict the prognosis of muscle invasive bladder cancer. Transl Androl Urol 12: 176-186, 2023.
26. Liu D, Xu W, Ding X, Yang Y, Su B and Fei K: Polymorphisms of CCNB1 associated with the clinical outcomes of platinum-based chemotherapy in Chinese NSCLC Patients. J Cancer 8: 3785-3794, 2017.
27. Liu A, Zeng S, Lu X, Xiong Q, Xue Y, Tong L, Xu W, Sun Y, Zhang Z and Xu C: Overexpression of G2 and S phase-expressed-1 contributes to cell proliferation, migration, and invasion via regulating p53/FoxM1/CCNB1 pathway and predicts poor prognosis in bladder cancer. Int J Biol Macromol 123: 322-334, 2019.
28. Chen Q, Hu J, Deng J, Fu B and Guo J: Bioinformatics analysis identified key molecular changes in bladder cancer development and recurrence. Biomed Res Int 16: 3917982, 2019.
29. Brcic L, Heidinger M, Sever AZ, Zacharias M, Jakopovic M, Fediuk M, Maier A, Quehenberger F, Seiwerth S and Popper H: Prognostic value of cyclin A2 and B1 expression in lung carcinoids. Pathology 51: 481-486, 2019.