Comprehensive analysis reveals the value of the expression of chromobox family members for bladder urothelial carcinoma prognosis

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Abstract. Bladder urothelial carcinoma (BLCA) accounts for 95% of all cases of bladder cancer worldwide, with a high incidence and poor prognosis. Chromobox (CBX) proteins play a key role in numerous malignant tumors; however, the role of CBX in BLCA remains unknown. Herein, the present study found that, compared with in normal bladder tissues, the expression levels of CBX1, CBX2, CBX3, CBX4 and CBX8 were markedly increased in BLCA tissues, as determined by Tumor Immune Estimation Resource, UALCAN and ONCOMINE analyses, whereas CBX6 and CBX7 were decreased in BLCA tissues. Furthermore, evident hypomethylation in the promoters of CBX1, and CBX2, as well as significant hypermethylation in the promoters of CBX5, CBX6 and CBX7, was detected in BLCA tissues compared with in normal bladder tissues. The expression of CBX1, CBX2 and CBX7 was involved in the prognosis of patients with BLCA. Low CBX7 expression was strongly associated with poorer overall survival in patients with BLCA, whereas high CBX1 and CBX2 expression was associated with poorer progression-free survival. Besides, significant associations were determined between the expression of CBXs and immune cell infiltration, including dendritic cells, neutrophils, macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells. Overall, the current results may provide a rationale for developing new targets and prognostic markers for BLCA therapy.

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Introduction

Approximately 500,000 new cases and 200,000 deaths from bladder cancer occur worldwide each year. The incidence of bladder cancer has gradually increased, causing a great burden on human health (1-3). Bladder urothelial carcinoma (BLCA) is the most common subtype of bladder cancer, accounting for >95% of cases (4). Although early BLCA diagnosis and treatment options have rapidly improved, the prognosis of patients with metastatic BLCA remains poor, with a 5-year survival rate of ~50% (2). In the past few decades, numerous studies have aimed to reveal the cause of BLCA pathogenesis; however, the underlying mechanisms are still unclear (5-7). Thus, more valuable markers for the prognosis and effective targets for BLCA therapy are urgently needed.

Chromobox (CBX) family members participate in chromosomal modification, transcriptional repression, cell differentiation and senescence (8), and eight CBX proteins have been described to date. These proteins are divided into two groups, including the Pc (containing N-terminal chromodomain) group and the HP1 (containing C-terminal and N-terminal chromodomains) group (9). Additionally, a recent study confirmed that CBXs are associated with a number of tumors (10). For example, CBX2 is significantly associated with colorectal cancer (CRC) prognosis because of its regulatory function in the apoptosis and proliferation of CRC cells (11). Furthermore, CBX4 can predict a worse prognosis in patients with hepatocellular carcinoma by promoting tumor angiogenesis (12) and CBX7 can suppress bladder cancer progression by modulating aldo-keto reductase family 1 member B10/ERK signaling (13). Nevertheless, whether the other CBXs participate in BLCA occurrence remains unknown. In the current study, the expression patterns and genetic alterations of CBXs and their association with the clinicopathological parameters and infiltration of immune cells in BLCA were analyzed using online public databases. The present results showed that CBXs participated significantly in BLCA pathogenesis and progression, and may be used as potential clinical therapeutic targets for BLCA therapy.

Materials and methods

UALCAN. UALCAN (http://ualcan.path.uab.edu) is a comprehensive cancer data website based on The Cancer Genome Atlas (TCGA) database (14,15). UALCAN was used to obtain data (TCGA-BLCA) on CBX mRNA expression and promoter methylation levels in BLCA tissues and normal tissues (from healthy controls), as well as the association between CBX expression and clinicopathological parameters. The beta value (percentage of methylation signal strength) is the most commonly used quantitative method for methylation levels. Differences in CBX mRNA expression and promoter methylation levels compared with the corresponding controls (from healthy controls) were analyzed by Welch's T-test, and $P<0.05$ was considered to indicate a statistically significant difference. Differences in CBX expression were compared among individual cancer stages by one-way ANOVA followed by the Dunnett's post hoc multiple comparisons test, and $P<0.05$ was considered to indicate a statistically significant difference.

ONCOMINE. Expression analyses were conducted using ONCOMINE (www.oncomine.org). An unpaired t-test was used to examine differential expression between the cancer and normal groups (from healthy controls), and the error detection rate was used as a correction measure of significance (16). Fold change and cut-off P-values were set as 1.5 and 0.01, respectively.

Cell culture. The SV-HUC-1 human ureteral epithelial immortalized cell line was obtained from the American Type Culture Collection and cultured in f12k (iCell Bioscience, Inc.) media with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2 in humidified air. The 5637 bladder cancer cell line was obtained from the American Type Culture Collection and cultured in 1640 media (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum at 37˚C with 5% CO_2 in humidified air. The UMUC3 bladder cancer cell line was obtained from the American Type Culture Collection and cultured in 1640 media with 10% fetal bovine serum at 37˚C with 5% CO_2 in humidified air. Media were not supplemented with antibiotics. The culture medium was replaced every 48 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA (from SV-HUC-1, 5637 and UMUC3 cells) was isolated using the total RNA extraction kit (Takara Bio, Inc.). The Bestar™ qPCR RT Kit (Takara Bio, Inc.) was used to synthesize cDNA from isolated total RNA, according to the manufacturer's protocol. Samples were processed in the Applied Biosystems 7500 Real-Time PCR System using TB Green Premix Ex Taq II (cat. no. RR820A; Takara Bio, Inc.). The reaction steps were as follows: i) Pre-denaturation, 95˚C for 30 sec; and ii) PCR reaction (40 cycles), 95˚C for 5 sec, 60˚C for 30 sec. The primer sequences for β-actin and CBXs are shown in Table I. Cycle threshold values were collected to calculate the relative expression of target genes, using the $2^{-\Delta\Delta Cq}$ method of quantification (17).

Western blotting. SV-HUC-1, 5637 and UMUC3 cells were lysed in NP-40 lysis buffer (cat. no. P0013F; Beyotime Institute of Biotechnology) containing protease inhibitors (Beyotime Institute of Biotechnology). After a 15 min of centrifugation at 15,000 x g at 4˚C, the supernatants were collected and protein concentration was assessed using a BCA assay (Thermo Fisher Scientific, Inc.) analyzed in a Multiskan full-wavelength microplate reader (Thermo Fisher Scientific, Inc.). After separation by SDS-PAGE on 10% gels (40 μg protein loaded per lane), proteins were transferred to a PVDF membrane and blocked for 1 h using 5% non-fat dry milk at room temperature. Subsequently, membranes were incubated with primary antibodies overnight at 4˚C, and then with the secondary antibody for 1 h at room temperature after three washes in TBS-Tween (containing 0.1% Tween). Primary antibodies were used at the following dilutions: CBX1 (cat. no. 10241-2-AP; Proteintech Group, Inc.) at 1:2,000, CBX2 (cat. no. 15579-1-AP; Proteintech Group, Inc.) at 1:3,000 and CBX7 (cat. no. 26278-1-AP; Proteintech Group, Inc.) at 1:1,000, and GAPDH (cat. no. 2118; Cell Signaling Technology, Inc.) at 1:2,000. The secondary antibody was HRP-linked anti-rabbit IgG (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.). The protein bands were visualized and semi-quantified using the Gel DOC XR Gel Imaging System and Image lab v5.2 software (Bio-Rad Laboratories, Inc.), respectively. *Human protein atlas (HPA).* The HPA (https://www.proteinatlas.org) was used to collect data about protein levels and distribution in BLCA and normal urothelial tissue immunohistochemistry (18). The immunohistochemical images of the expression of CBXs in BLCA and normal tissues (from healthy controls) were extracted from the HPA.

Survival analysis. To investigate the prognostic value of CBXs, patient data from the Kaplan-Meier (KM) plotter (http://kmplot.com; dataset name, BLCA; mRNA; 0.05 significance cut-off value) were classified into two groups according to the median expression value of CBX, and the practicality in determining patient prognosis was evaluated. A number of R (version 3.6.1) packages were used for statistical computations and for generating the output graphs (19-22). The 'survival' package was used for univariate and multivariate KM analyses (21). Survival curve plots were generated using the 'survplot' package. The 'XML' and 'rjson' R packages were used to load configuration files, the 'RODBC' package was used to communicate with the database, and the 'ggplot2' package was used to visualize the results (19,20,22). The significance was computed using the Cox-Mantel test to compare two cohorts. The difference between cohorts was numerically characterized by the hazard rate (HR) based on the differential descent rate of the two cohorts. Since the HR is a comparison to the baseline by definition, a relative two-fold drop in one cohort equals a half-fold drop in the other cohorts. Thus, depending on the context, an HR of 2 equals an HR of 0.5. As it is easier to understand an HR above 1 in most cases, an option to invert all HR values below 1 was implemented (23,24). $P<0.05$ was considered to indicate a statistically significant difference.

Gene expression profiling interactive analysis (GEPIA). GEPIA (http://gepia.cancer-pku.cn/index.html) is a website for gene expression profiling analysis. GEPIA generates a list of genes with similar expression patterns ranked by Pearson correlation coefficient (25). Here, GEPIA was used to select the first 20

Table I. Primer sequences of CBX family members.

Gene	Species	Forward	Reverse
β-actin	Human	5-TCTCCCAAGTCCACACAGG-3	5-GGCACGAAGGCTCATCA-3
CBX1	Human	5-GAGCCGGAGCGGATTATTGG-3	5-GGGCACTTGACATTGGCTTC-3
CBX2	Human	5-TCCTGGCCTTCCAGAAGAAG-3	5-CAGGAGGACATGGCAGTGAG-3
CBX3	Human	5-TTGAAGAGGCAGAGCCTGAA-3	5-AGGTTCCCAAGTATTGTCAGC-3
CBX4	Human	5-TGATCGCCTTCCAGAACAGG-3	5-TCAGTGGAGGAGTCCTGGAG-3
CBX6	Human	5-CGAAAGGGACGCATCGAGTA-3	5-GCGAGTCCAGGATGTTCTCC-3
CBX7	Human	5-TGCGGAAGGGTAAAGTCGAG-3	5-TCCTCCTTCTCCTCGTAGGC-3
CBX8	Human	5-GATCCCTGTGGCCAGAATCC-3	5-GTGACCACCACCTTCTCCAG-3

CBX, chromobox.

genes in BLCA that were similar to the CBX family; a total of 129 genes were identified for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (26).

Metascape. The Metascape database (http://metascape.org) was used for enrichment analysis. The express analysis module in Metascape was used for GO and KEGG analysis of CBX and similar genes (26). The relationship between physical proteins captured in BioGrid is the main data source of Metascape (27). Metascape automatically extracts protein-protein interaction networks from these candidates. Then, for each connected network component, the MCODE algorithm is applied to identify the complex of dense connections (28), and Cytoscape is used for visualization (29).

cBioPortal. cBioPortal (www.cbioportal.org) is an online open access website resource for analyzing cancer genomics data (30). In the present study, gene mutations of CBXs in BLCA were analyzed using this resource.

Tumor immune estimation resource (TIMER). TIMER (https://cistrome.shinyapps.io/timer/) is a website for analyzing immune cell infiltration into tumor tissues (31). The present study used the Gene and Survival modules in TIMER to analyze the link between the expression of CBXs and immune cell infiltration, as well as the association of immune cell infiltration with the clinical outcome of patients with BLCA. The 'Gene' module was used to explore the correlation between CBXs and the level of immune infiltration in BLCA. Scatterplots were generated, showing the purity-corrected partial Spearman's ρ value and statistical significance. The 'DiffExp' module allows the study of differential expression between BLCA tumor and adjacent normal tissues (adjacent tissues from the same patients) for the CBX genes across the BLCA dataset from TCGA. The Cox proportional hazard model ('survival' module) was used to explore the clinical relevance of BLCA tumor immune cell subsets and CBX expression levels. The distributions of gene expression levels are displayed using box plots, with the statistical significance of differential expression evaluated using the Wilcoxon test. Up or downregulated genes in tumors compared with in normal tissues (adjacent tissues from the same patients) can be identified for each cancer type.

Statistical analysis. All collected data were analyzed using SPSS (version 20.0; IBM Corp.) and are expressed as the mean ± SEM. *In vitro* experiments were repeated $^{3}3$ times. The difference between indicated groups was evaluated by Student's t-test, Welch's T-test, Wilcoxon test or ANOVA. Unpaired Student's t-test (parametric) or Wilcoxon signed-rank test (non-parametric) were used to compare the datasets of two groups. One-way ANOVA (parametric) were used to compare the datasets of multiple groups. If three datasets were analyzed, the LSD post hoc multiple comparisons test was performed following ANOVA; otherwise, the Dunnett's post hoc multiple comparisons test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CBXs and methylation of their promoters in patients with BLCA. The UALCAN, TIMER and ONCOMINE databases were used to evaluate the expression of CBXs in patients with BLCA. Compared with in normal bladder mucosa tissues, BLCA tissues exhibited markedly elevated expression of CBX1 (Figs. 1A and 2), CBX2 (Figs. 1B, 2 and 3), CBX3 (Figs. 1C, 2 and 3), CBX4 (Figs. 1D and 2) and CBX8 (Figs. 1H and 2), and reduced expression of CBX6 (Figs. 1F, 2 and 3) and CBX7 (Figs. 1G, 2 and 3). However, no significant difference was detected for CBX5 (Figs. 1E, 2 and 3) between BLCA and normal bladder mucosa tissues. Moreover, the expression of CBXs in normal SV-HUC-1 bladder cells, and 5637 and UMUC3 bladder tumor cells was evaluated by RT-qPCR. Consistent with the aforementioned results, elevated expression levels of CBX1 (Fig. 4A), CBX2 (Fig. 4B), CBX3 (Fig. 4C), CBX4 (Fig. 4D) and CBX8 (Fig. 4G), and reduced expression levels of CBX6 (Fig. 4E) and CBX7 (Fig. 4F) were detected in BLCA cells compared with in normal SV-HUC-1 cells. These results were also supported by the expression of CBXs at the protein level determined using the HPA (Fig. 5). Additionally, promoter methylation was associated with the progression of bladder tumors. Hypermethylation was detected in the promoters of CBX5 (Fig. 6E), CBX6 (Fig. 6F) and CBX7 (Fig. 6G), whereas hypomethylation was identified in the promoters of CBX1 (Fig. 6A) and CBX2 (Fig. 6B).

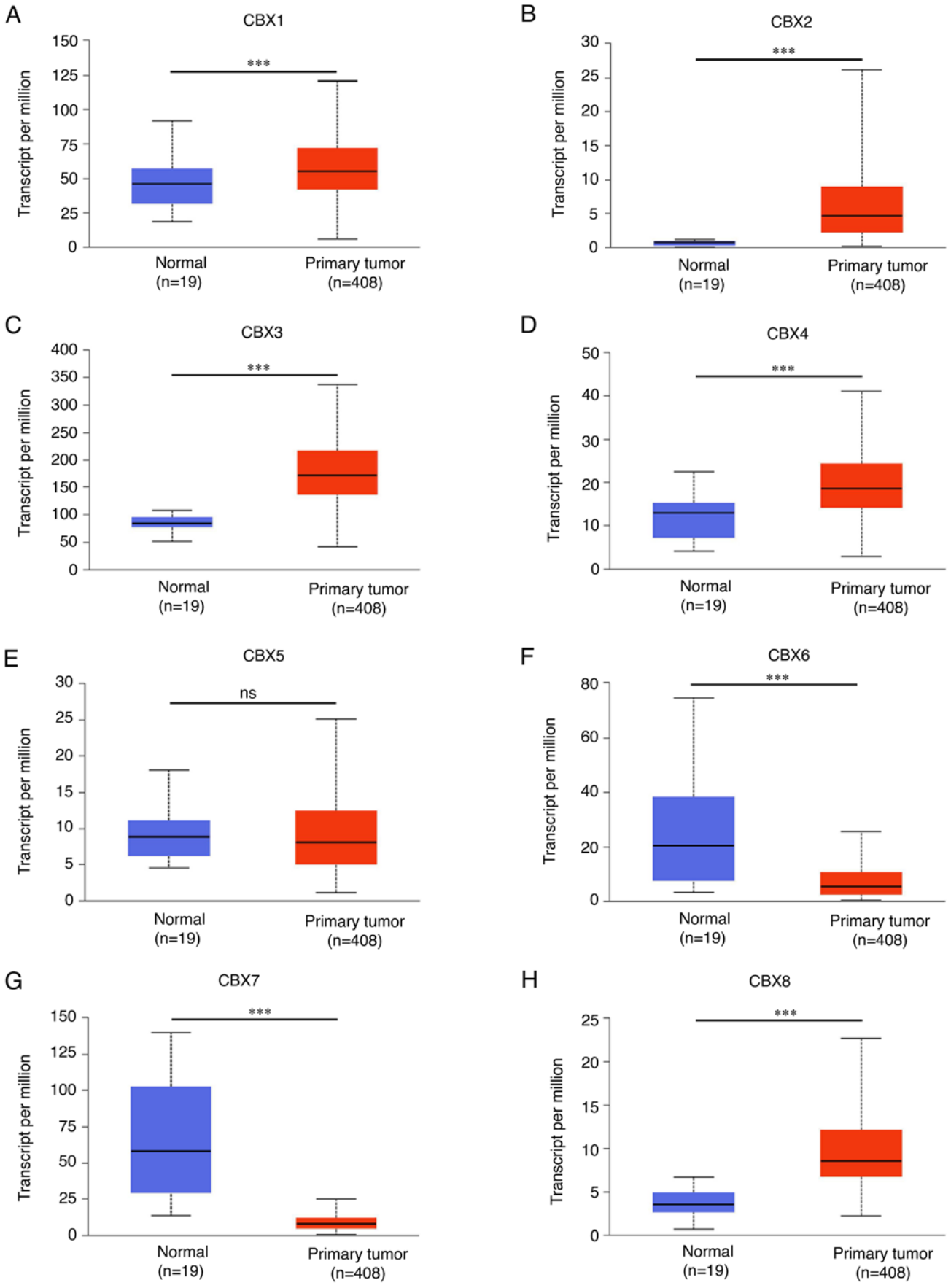

Figure 1. mRNA expression levels of CBX family members in BLCA and normal (from healthy controls) bladder tissues, as determined using UALCAN. The mRNA expression levels of (A) CBX1, (B) CBX2, (C) CBX3 and (D) CBX4 were increased in BLCA tissues compared with in normal bladder tissues. (E) mRNA expression levels of CBX5 did not change. The mRNA expression levels of (F) CBX6 and (G) CBX7 were reduced in BLCA tissues compared with in normal bladder tissues. The mRNA expression levels of (H) CBX8 were increased in BLCA tissues compared with in normal bladder tissues. The mRNA expression differences were compared by Welch's T-test. ***P<0.001. ns, no significance; BLCA, bladder urothelial carcinoma; CBX, chromobox.

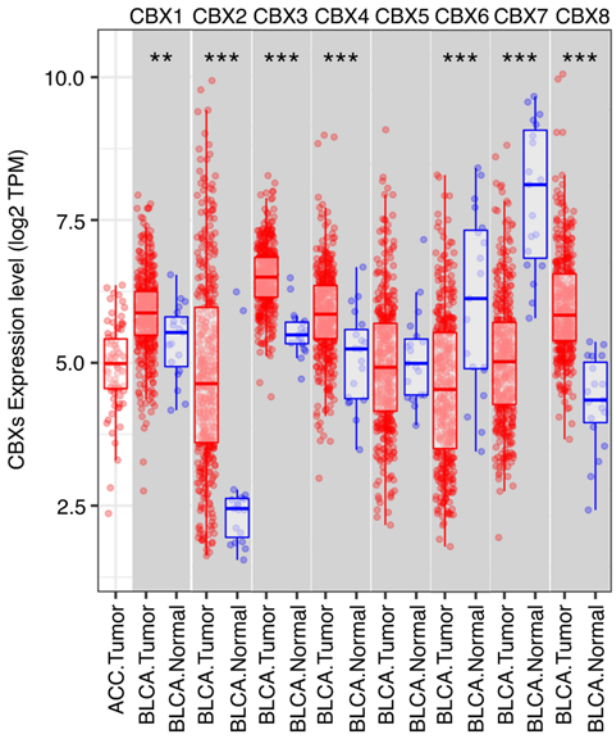

Figure 2. mRNA expression levels of CBXs in BLCA, as determined using TIMER. The mRNA expression levels of CBX1, CBX2, CBX3, CBX4 and CBX8 were increased in BLCA tissues compared with in normal (adjacent tissues from the same patients) bladder tissues, whereas CBX6 and CBX7 were reduced. CBX5 expression was unaltered. Distributions of gene expression levels are displayed using box plots, with the statistical significance of differential expression evaluated by the Wilcoxon signed-rank test. TPM represent the relative abundance of a transcript among a population of sequenced transcripts. **P<0.01, ***P<0.001. ns, no significance; TPM, transcripts per million; BLCA, bladder urothelial carcinoma; CBX, chromobox; TIMER, Tumor Immune Estimation Resource.

Association between CBXs and the cancer stage of patients with BLCA. The cancer stage was negatively associated with the expression of CBX6 (Fig. 7F) and CBX7 (Fig. 7G), whereas it was positively associated with CBX1 (Fig. 7A), CBX2 (Fig. 7B), CBX3 (Fig. 7C), CBX4 (Fig. 7D) and CBX8 (Fig. 7H). Notably, no association was identified between CBX5 (Fig. 7E) expression and the cancer stages of patients. Hence, it was indicated that CBX1, CBX2, CBX3, CBX4, CBX6, CBX7 and CBX8 expression levels were closely associated the stages of patients with BLCA.

Network and functional enrichment analyses of the CBX family and their neighboring genes in patients with BLCA. The biological classification of CBXs was investigated via pathway and function enrichment analyses using Metascape. The GO and KEGG analyses predicted the functions of CBX family genes according to the enrichment of CBXs. The enrichment items were divided into four functional groups: Cellular components (Fig. 8A and B), molecular functions (Fig. 8C and D), biological processes (Fig. 8E and F), and KEGG pathways (Fig. 8G and H). CBX and their related genes were enriched for: The 'mitotic cell cycle', 'RNA splicing', 'cell division', 'PcG protein complex', 'chromatin binding', 'nuclear matrix', 'single-stranded RNA binding', 'single-stranded DNA binding', 'nuclear chromosome', 'spliceosome', 'herpes simplex virus 1 infection', 'apoptosis' and 'amyotrophic lateral sclerosis'.

Value of CBXs for the prognosis of patients with BLCA. BLCA patients were ranked according to CBXs expression. Half of the patients with high CBXs expression were included in the high expression group, and the second half of the patients with low CBXs expression were included in the low expression group. Patients with high expression of CBX1 (Fig. 9B), CBX2 (Fig. 9D), CBX5 (Fig. 9J) and CBX6 (Fig. 9L) exhibited poor progression-free survival (PFS), whereas patients with low expression of CBX3 (Fig. 9E) and CBX7 (Fig. 9M) exhibited poor overall survival (OS). Patients with high expression of CBX6 (Fig. 9K) exhibited poor OS. These results suggested that CBX1, CBX2 and CBX7 expression levels were closely related to the prognosis of patients with BLCA. There was no significant difference in OS between patients with high and low expression of CBX1 (Fig. 9A), CBX2 (Fig. 9C), CBX4 (Fig. 9G), CBX5 (Fig. 9I) and CBX8 (Fig. 9O). Additionally, there was no significant difference in PFS between patients with high and low expression of CBX3 (Fig. 9F), CBX4 (Fig. 9H), CBX7 (Fig. 9N) and CBX8 (Fig. 9P).

Protein levels of CBXs with prognostic value in different cell lines. The protein expression levels of CBX1 were significantly increased in 5637 and UMUC3 cells compared with in SV-HUC-1 cells (Fig. 10B). Similarly, the protein expression levels of CBX2 were significantly increased in UMUC3 cells compared with in SV-HUC-1 cells (Fig. 10C). By contrast, the protein expression levels of CBX7 were significantly decreased in UMUC3 cells compared with in SV-HUC-1 cells (Fig. 10D).

Genetic alterations of CBXs in patients with BLCA. The genetic alterations of CBXs in patients with BLCA were analyzed using the cBioPortal online tool. The results indicated that generally, 48% of patients with BLCA exhibited CBX1-8 genetic alterations. The OncoPrints include missense mutations, truncating mutations, structural variants, amplification, deep deletion, and abnormal expressions. The genetic alteration rates for CBX1-8 were 10, 8, 10, 9, 10, 9, 10 and 8%, respectively (Fig. 11).

Infiltration of immune cells in patients with BLCA. Positive correlations were observed between CBX1 and CBX3 expression and the infiltration of dendritic cells, neutrophils, macrophages and $CD8^+$ T cells (Fig. 12A and C); CBX2 was positively correlated with macrophage infiltration (Fig. 12B); CBX4 was positively correlated with neutrophil, macrophage and B cell infiltration (Fig. 12D); CBX5 was positively correlated with the infiltration of dendritic cells, neutrophils, macrophages, $CD4^+$ and $CD8^+$ T cells (Fig. 12E); CBX6 was positively correlated with macrophage, $CD4^+$ T cell, $CD8^+$ T cell and B cell infiltration (Fig. 12F); CBX7 was positively correlated with the infiltration of macrophages, $CD4^+$ T cells and B cells (Fig. 12G); and CBX8 was positively correlated with macrophage infiltration (Fig. 12H). Meanwhile, negative correlations were observed between CBX7 expression and the infiltration of dendritic cells and $CD8^+$ T cells (Fig. 12G); and CBX8 with dendritic cell and neutrophil infiltration (Fig. 12H). The present results also found that macrophages, CBX6 and CBX7 were associated with the clinical outcome of patients

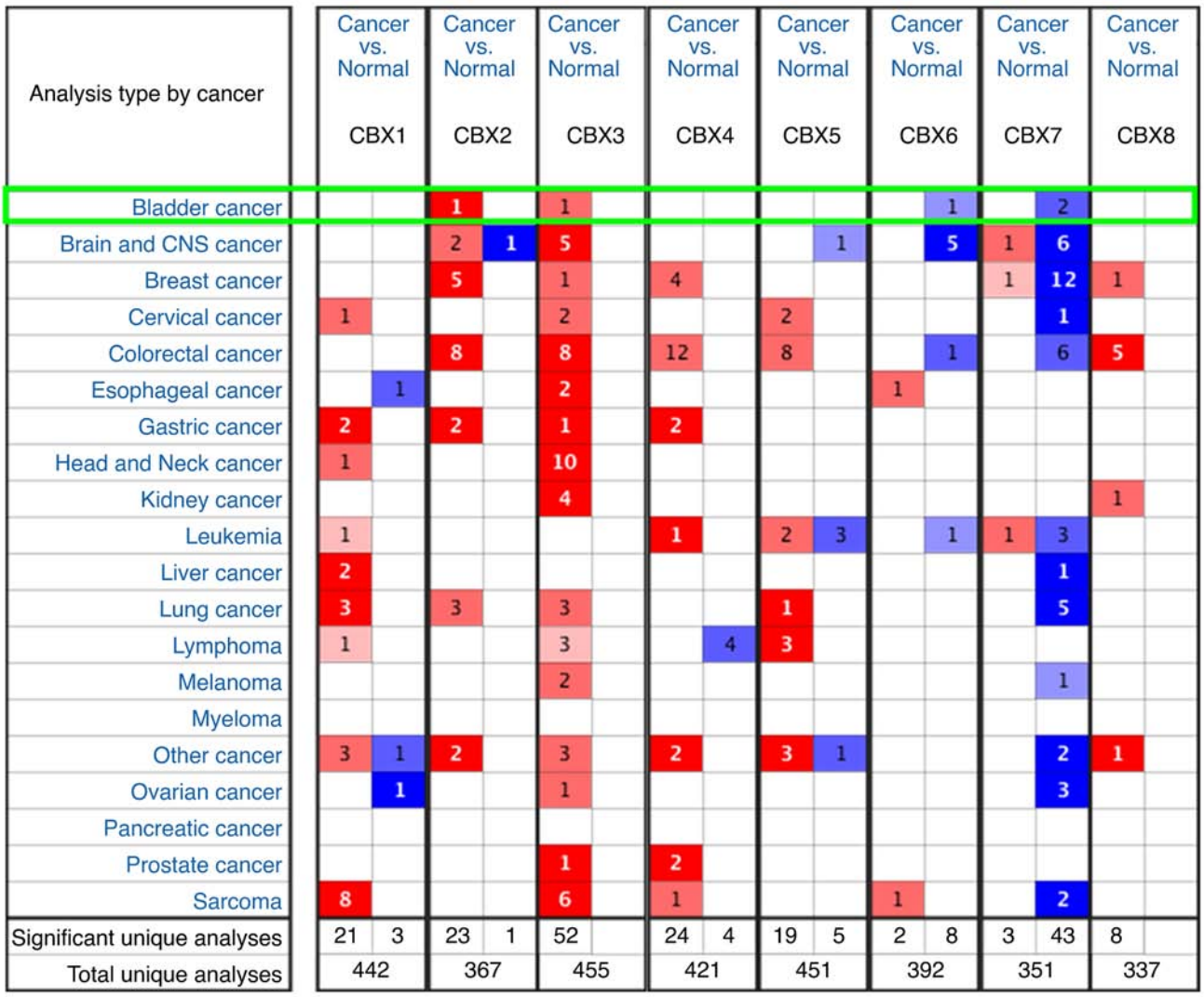

Figure 3. mRNA expression levels of CBXs in different types of cancer analyzed using ONCOMINE. The mRNA expression differences were compared by unpaired Student's t-test. The cut-off values were: P<0.01; FC, 1.5; gene rank, 10%; data type, mRNA. CBX, chromobox; FC, fold change.

with BLCA (Table II). However, all correlation values were <0.3 or >-0.3, which indicates that they were weak.

Discussion

BLCA progression is considered a long-term, multi-step process. Besides genetics, studies have also indicated that epigenetic regulation participates in BLCA progression (32,33). CBX family proteins are important components of epigenetic regulatory complexes and are involved in chromatin modification, and the pathogenesis and progression of a number of tumors (10). Nevertheless, the function of CBXs in BLCA development and patient prognosis remains unclear. Herein, the present study analyzed the expression, the prognostic value and mutation of CBXs in BLCA samples, and showed that seven CBXs were differentially expressed in BLCA samples. Furthermore, the expression of CBX1, CBX2 and CBX7 was associated with the prognosis of patients with BLCA. Besides, the expression of six CBXs was related to the cancer stages of BLCA. In addition, significant associations between CBX expression and the infiltration of six immune cells and the prognosis of patients with BLCA were observed. Moreover, abnormal expression of CBXs in BLCA could be caused by promoter methylation and genetic mutations. To the best of our knowledge, this was the first study to comprehensively analyze the significance of CBXs in BLCA progression.

A number of studies have demonstrated high CBX1 expression in numerous tumor tissues, such as gastric carcinoma (34), castration-resistant prostate cancer (PCa) (35), hepatocellular carcinoma (36), ovarian carcinoma (37) and pituitary carcinoma (38). In PCa, CBX1 overexpression has been reported to be involved in trimethylation levels of histone H3K9 and higher Gleason score. Impairment of CBX1 expression can reduce androgen receptor expression, inhibiting the proliferation of PCa cells (35). CBX1 also has reduced expression in renal clear cell carcinoma, and is related to tumor grade, tumor stage and patient prognosis (39). Nevertheless, the role of CBX1 in BLCA is poorly understood. Herein, the present study found that CBX1 expression was increased in BLCA tissues. Also, elevated CBX1 expression was related to patient tumor stages. Hence, the present results indicated that CBX1 may serve an oncogenic role in BLCA.

Previous studies have demonstrated that CBX2 has higher expression in various tumors than in normal tissues (40-42).

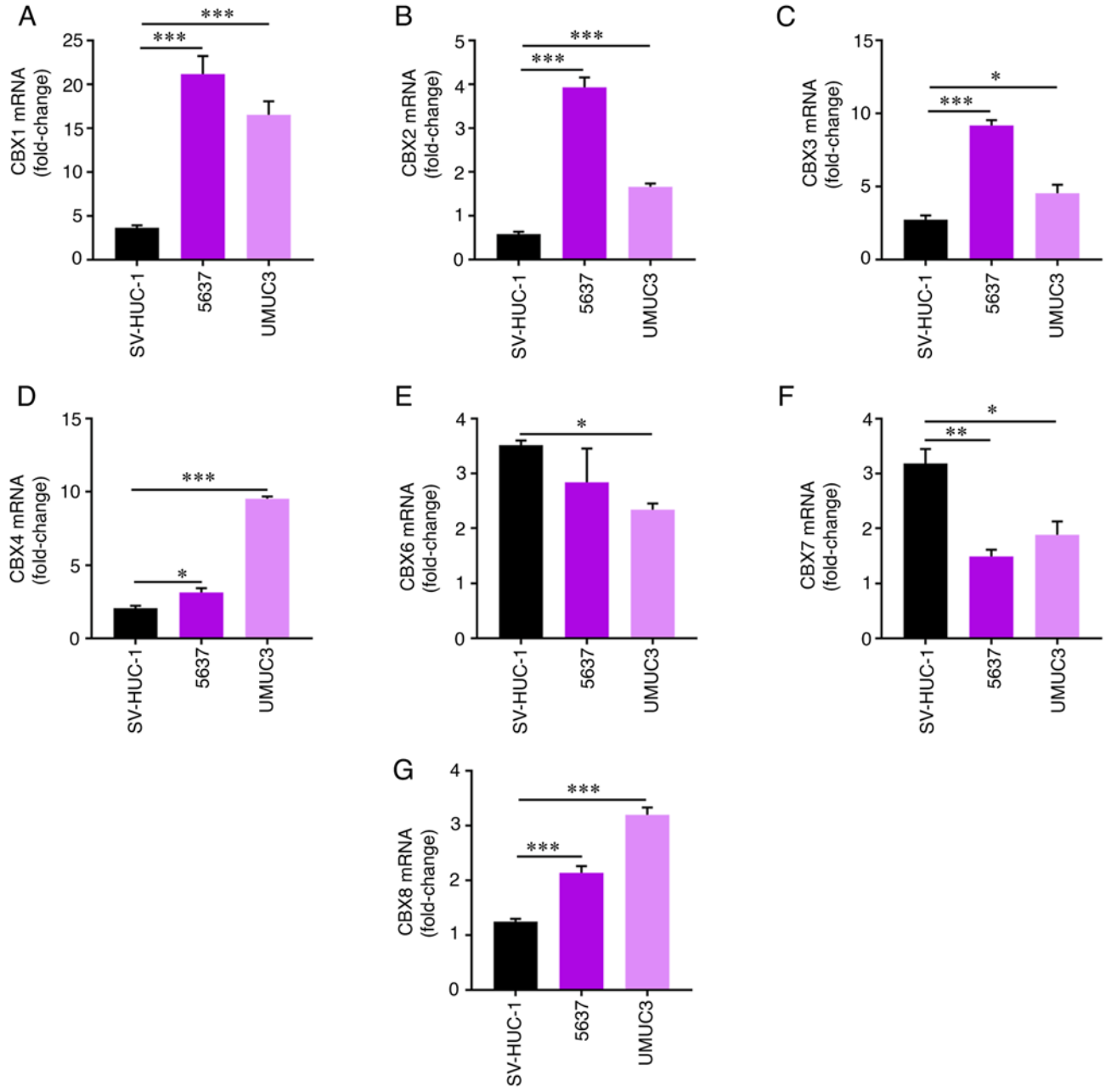

Figure 4. mRNA expression levels of CBXs in normal bladder mucosal cells and BLCA cells, as determined by reverse transcription-quantitative PCR. The mRNA expression levels of (A) CBX1, (B) CBX2, (C) CBX3 and (D) CBX4 were significantly increased in BLCA cells compared with in normal bladder mucosa cells, whereas (E) CBX6 and (F) CBX7 were reduced. (G) The mRNA expression levels of CBX8 were significantly increased in BLCA cells compared with in normal bladder mucosa cells. The difference among groups was evaluated by one-way ANOVA followed by the LSD post hoc multiple comparisons test. n≥3, *P<0.05, **P<0.01, ***P<0.001. BLCA, bladder urothelial carcinoma; CBX, chromobox.

For example, Hu *et al* (41) reported that CBX2 and enhancer of zeste homolog 2 (EZH2) synergistically promoted lung cancer progression, combining with promoters of the PPAR signaling pathway and tumor suppressor genes, cooperatively or individually, to downregulate their expression. Moreover, CBX2 overexpression can promote breast cancer progression by activating the PI3K/AKT signaling pathway (42). Additionally, cancer susceptibility candidate 9 promotes bladder cancer progression by regulating CBX2 (43). The present study detected significantly elevated CBX2 expression in BLCA and a connection with tumor stages. CBX2 overexpression was closely associated with poor PFS in patients with BLCA, suggesting the promotive effect of CBX2 on BLCA.

Significant CBX3 upregulation has been found in various tumors, such as PCa (44), gastric cancer (45) and lung cancer (46,47). Smoking-related upregulation of CBX3 promotes lung adenocarcinoma progression by forming a complex with tripartite motif-containing (TRIM)28, TRIM24 and RBBP4, suppressing Rho GTPase-activating protein 24 expression and increasing active Rac1 in lung adenocarcinoma cells (47). Huang *et al* (48) showed that LINC00857 is involved in the proliferation and development of diffuse large B-cell lymphoma by regulating the microRNA370-3p/CBX3 axis. However, another study has shown that the long non-coding RNA, LINC00998, can inhibit the development of malignant glioma by regulating the c-Met/Akt/mTOR signaling pathway

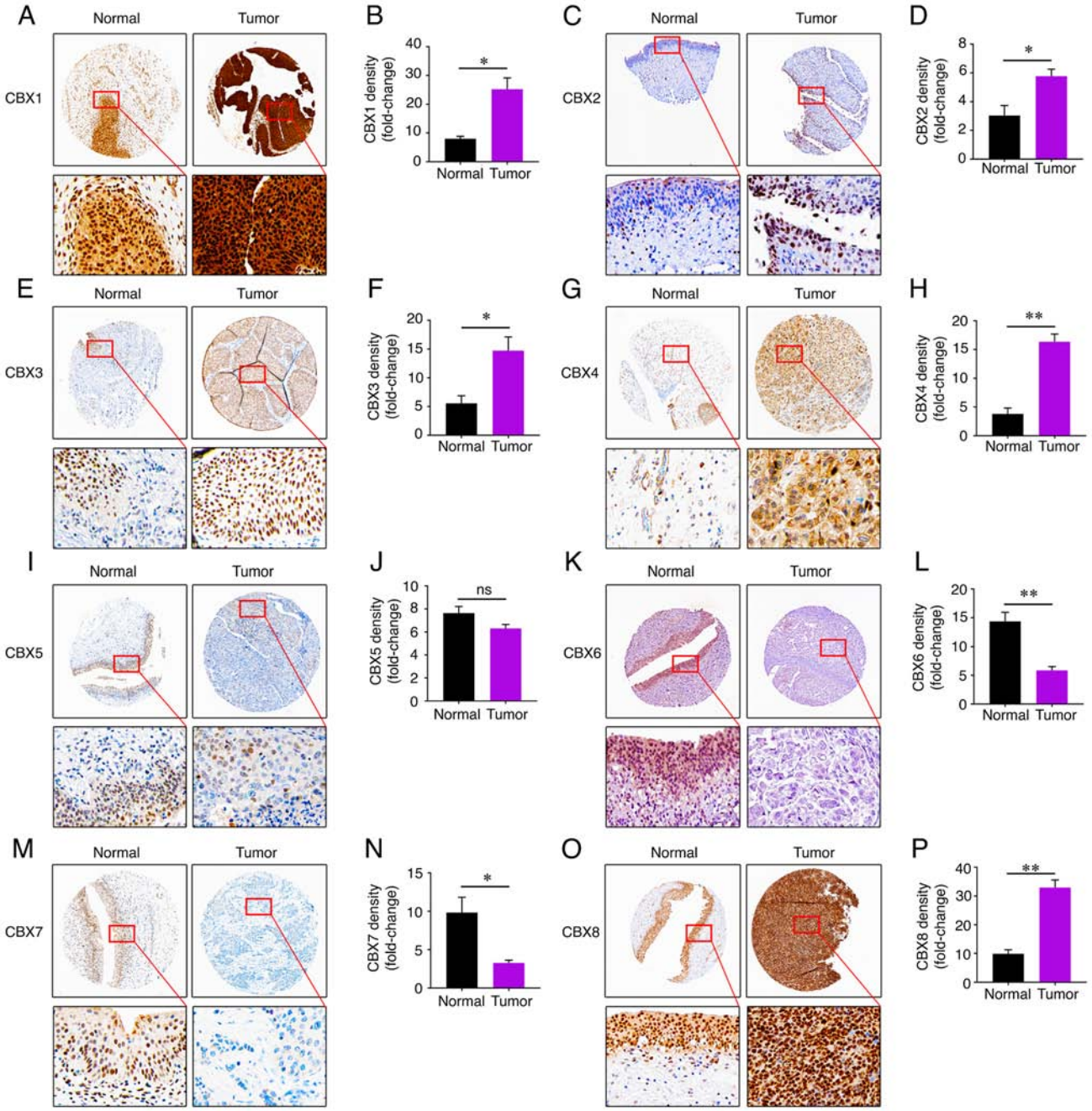

Figure 5. Protein levels of CBXs in normal (from healthy controls) bladder mucosal and BLCA tissues from the Human Protein Atlas. The protein levels of (A and B) CBX1, (C and D) CBX2, (E and F) CBX3, and (G and H) CBX4 were increased in BLCA tissues compared with in normal bladder tissues, whereas (I and J) CBX5 levels were unaltered. The protein levels of (K and L) CBX6 and (M and N) CBX7 were reduced in BLCA tissues compared with in normal bladder tissues, whereas those of (O and P) CBX8 were increased. Original magnification of the upper panels was x50. Original magnification of the lower panels was x400. The difference between groups was evaluated by unpaired Student's t-test. *P<0.05, **P<0.01. BLCA, bladder urothelial carcinoma; CBX, chromobox.

to prevent CBX3 ubiquitination and degradation (49). These studies suggested that CBX3 could be an oncogene or a tumor suppressor gene, depending on the tumor type. Herein, CBX3 expression was significantly increased in BLCA tissues and was associated with tumor stages. However, low expression of CBX3 was significantly related to poorer OS in patients with BLCA, which is in contradiction with the high expression of CBX3 observed in BLCA. Therefore, the role of CBX3 in BLCA is not clear.

CBX4 is upregulated in various human tumors, such as osteosarcoma (50), lung adenocarcinoma (51) and breast cancer (52). Lin *et al* (45) showed that patients with gastric cancer and with high CBX4 expression had poor prognoses. Wang *et al* (50) reported that CBX4 overexpression in osteosarcoma promoted tumor metastasis by transcriptionally upregulating RUNX2 and recruiting GCN5 to the RUNX2 promoter. Overexpressed CBX4 can promote cancer cell migration by regulating CDK2, cyclin E, MMP2, MMP9 and CXCR4 (51). However, Wang *et al* (53) reported that CBX4 inhibited CRC migration, invasion and metastasis by suppressing RUNX2 expression. Herein, the present study found that

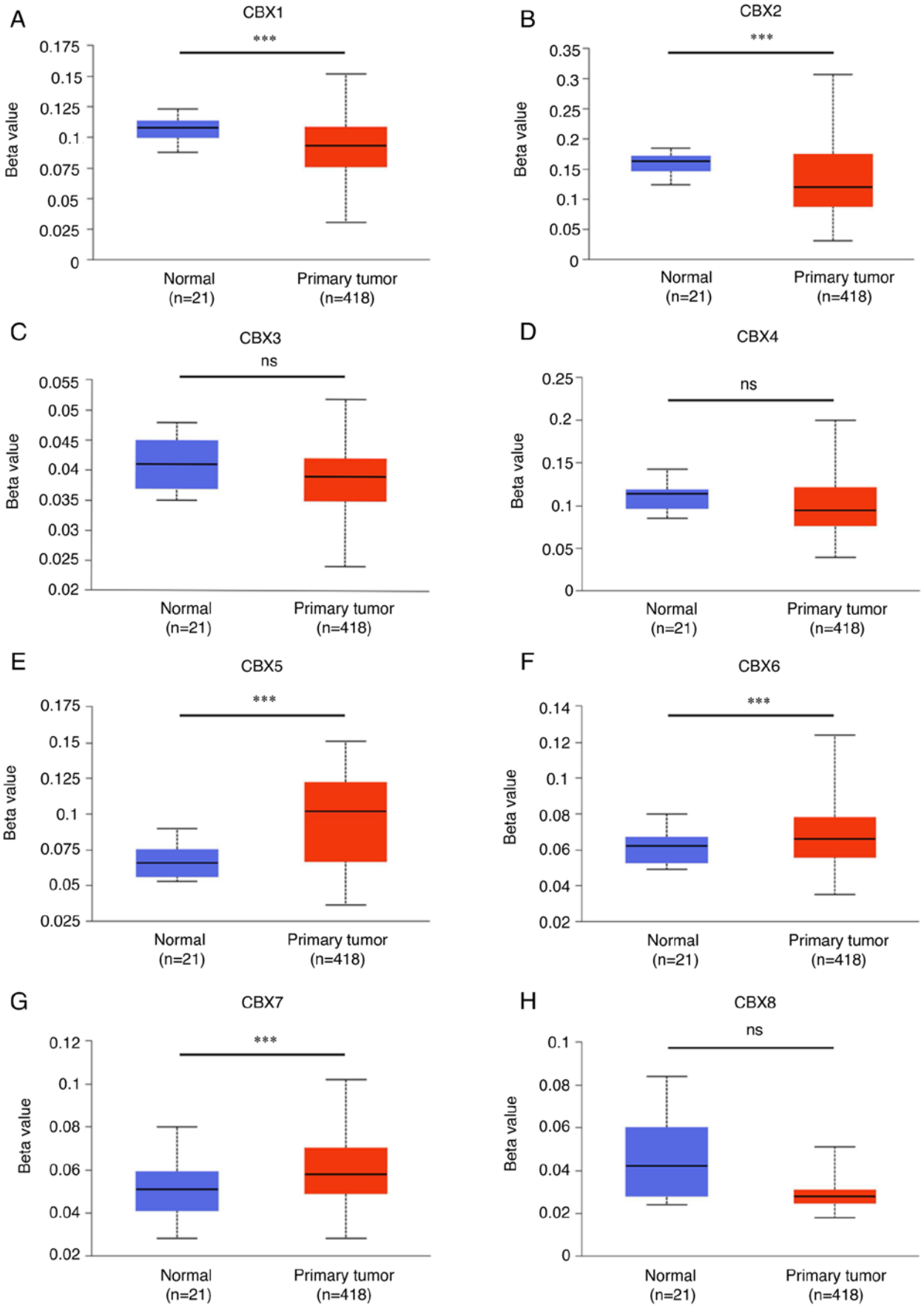

Figure 6. Promoter methylation status of CBXs in BLCA using UALCAN. The promoters of (A) CBX1 and (B) CBX2 were hypomethylated in BLCA tissues. The promoter methylation level of (C) CBX3 and (D) CBX4 did not change. The promoters of (E) CBX5, (F) CBX6 and (G) CBX7 were hypermethylated in BLCA tissues, whereas (H) CBX8 promoter methylation did not change. The beta value (percentage of methylation signal strength) is the most commonly used quantitative method of methylation levels. The differences in promoter methylation levels were compared by Welch's t-test. ***P<0.001. BLCA, bladder urothelial carcinoma; CBX, chromobox.

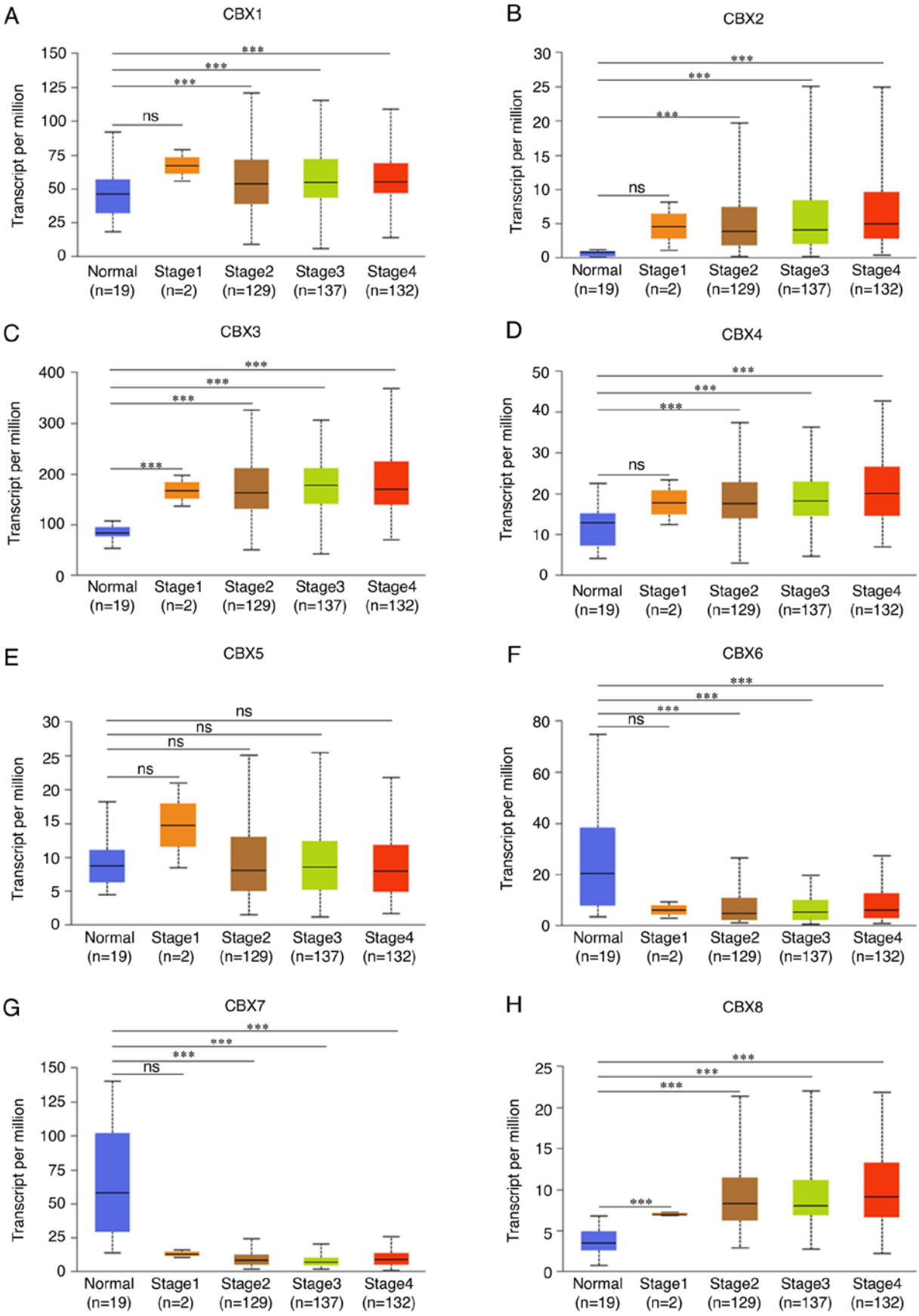

Figure 7. Association between mRNA expression levels of CBXs and tumor stages in patients with bladder urothelial carcinoma using UALCAN. The cancer stage was positively associated with the expression of (A) CBX1, (B) CBX2, (C) CBX3 and (D) CBX4, whereas it was not related to (E) CBX5. The cancer stage was negatively associated with the expression of (F) CBX6 and (G) CBX7, but it was positively associated with the expression of (H) CBX8. Stage 1, noninvasive papillary carcinoma, carcinoma *in situ* and tumor invades subepithelial connective tissue; Stage 2, tumor invades muscle; Stage 3, tumor invades perivesical tissue; Stage 4, tumor invades prostate, uterus, vagina, pelvic wall or abdominal wall. The difference among groups was evaluated by one-way ANOVA followed by the Dunnett's post hoc multiple comparisons test. ***P<0.001. ns, no significance; CBX, chromobox.

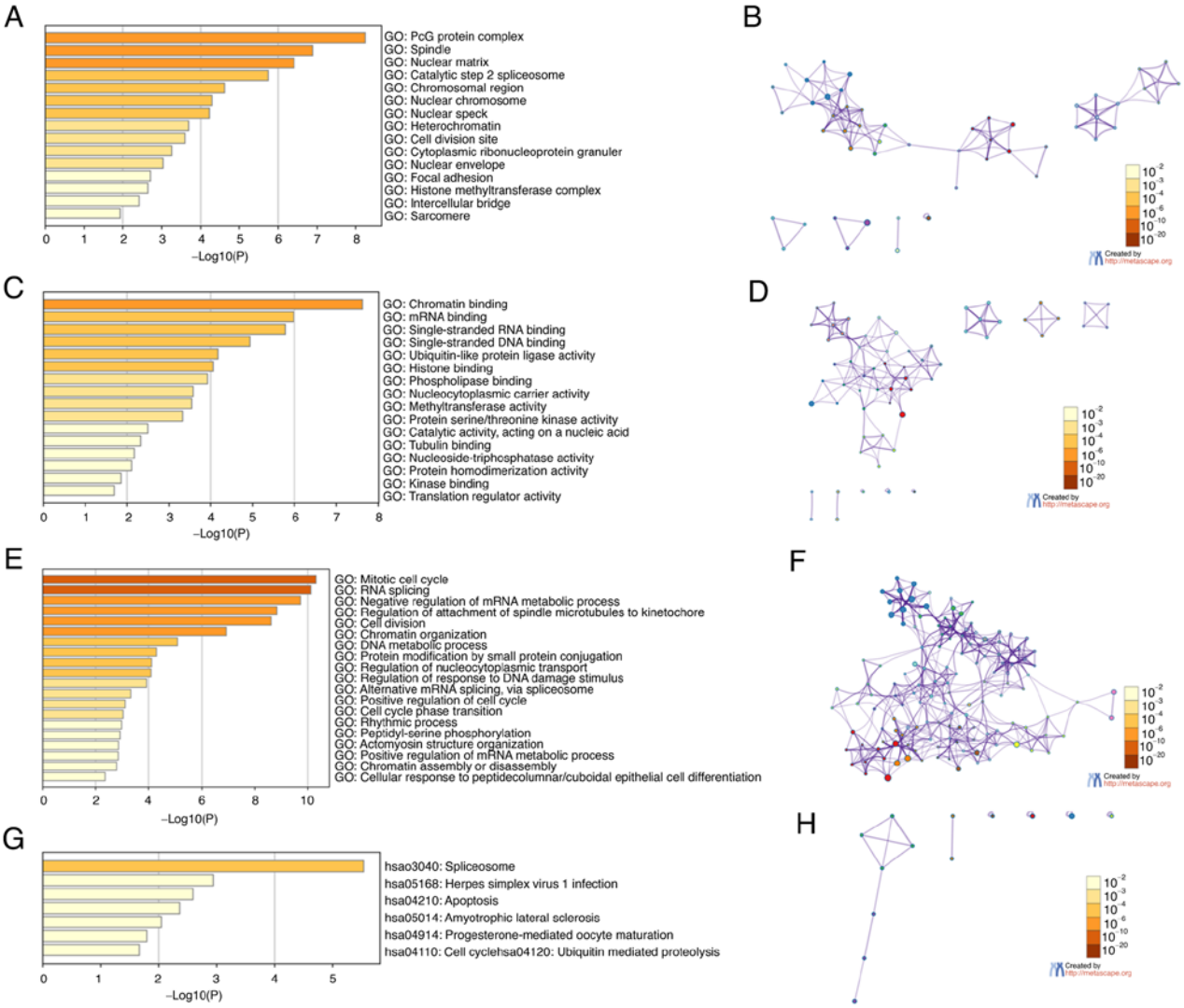

Figure 8. Enrichment analysis of chromobox family members and neighboring genes in BLCA using Metascape. (A) GO enrichment analyses of cellular components of CBXs similar genes. (B) Network of enriched cellular component terms colored by P-value. (C) GO enrichment analyses of molecular functions of CBXs similar genes. (D) Network of enriched molecular function terms colored by P-value. (E) GO enrichment analyses of biological processes of CBXs similar genes. (F) Network of enriched biological process terms colored by P-value. (G) KEGG enrichment analyses of KEGG pathways of CBXs similar genes. (H) Network of enriched KEGG pathways terms colored by P-value. GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

CBX4 expression was higher in BLCA tissues, and CBX4 was related to tumor stages, suggesting that it plays a pro-tumor role in BLCA.

CBX5 is expressed in various types of cancer, including renal and gastric cancer (54). Sun *et al* (55) reported that LINC02381 can promote CBX5 transcription through the interaction with CEBPβ, exerting a tumorigenic effect on glioma cells. Nevertheless, the present results suggested that CBX5 might not affect BLCA.

Conflicting roles of CBX6 have been found in tumors. For example, CBX6 overexpression can promote liver cancer progression by regulating the NF-κB/MAPK pathway (56). By contrast, Deng *et al* (57) reported that CBX6 was negatively regulated by EZH2 and exerted a tumor suppressor role in breast cancer; CBX6 was shown to inhibit breast cancer development by interacting with EZH2. Additionally, Sakai *et al* (58) reported that CBX6 proteasomal degradation promoted MMP-2 expression to induce mesothelioma progression. In the present study, CBX6 expression was associated with BLCA stage. Overall, the current findings suggested that CBX6 plays an anti-tumor role in BLCA.

Notably, CBX7 shows opposing effects in different tumors (59,60). Previous studies have shown that CBX7 is decreased in bladder cancer, cervical cancer and CRC, and its downregulation is related to poor survival in patients with cancer. These results suggested that CBX7 has a tumor suppressor role in these cancers (13,61-63). Conversely, CBX7 is overexpressed in ovarian cancer and PCa, and is related to poor prognosis, suggesting that increased CBX7 expression exhibits a cancer-promoting role in these tumors (64,65). The present study demonstrated that CBX7 levels were significantly reduced in BLCA tissues and that low CBX7 expression was positively related to the cancer stage of patients with BLCA. Furthermore, reduced levels of CBX7 were associated with poor prognoses, suggesting that it exerts an anti-tumor role in BLCA.

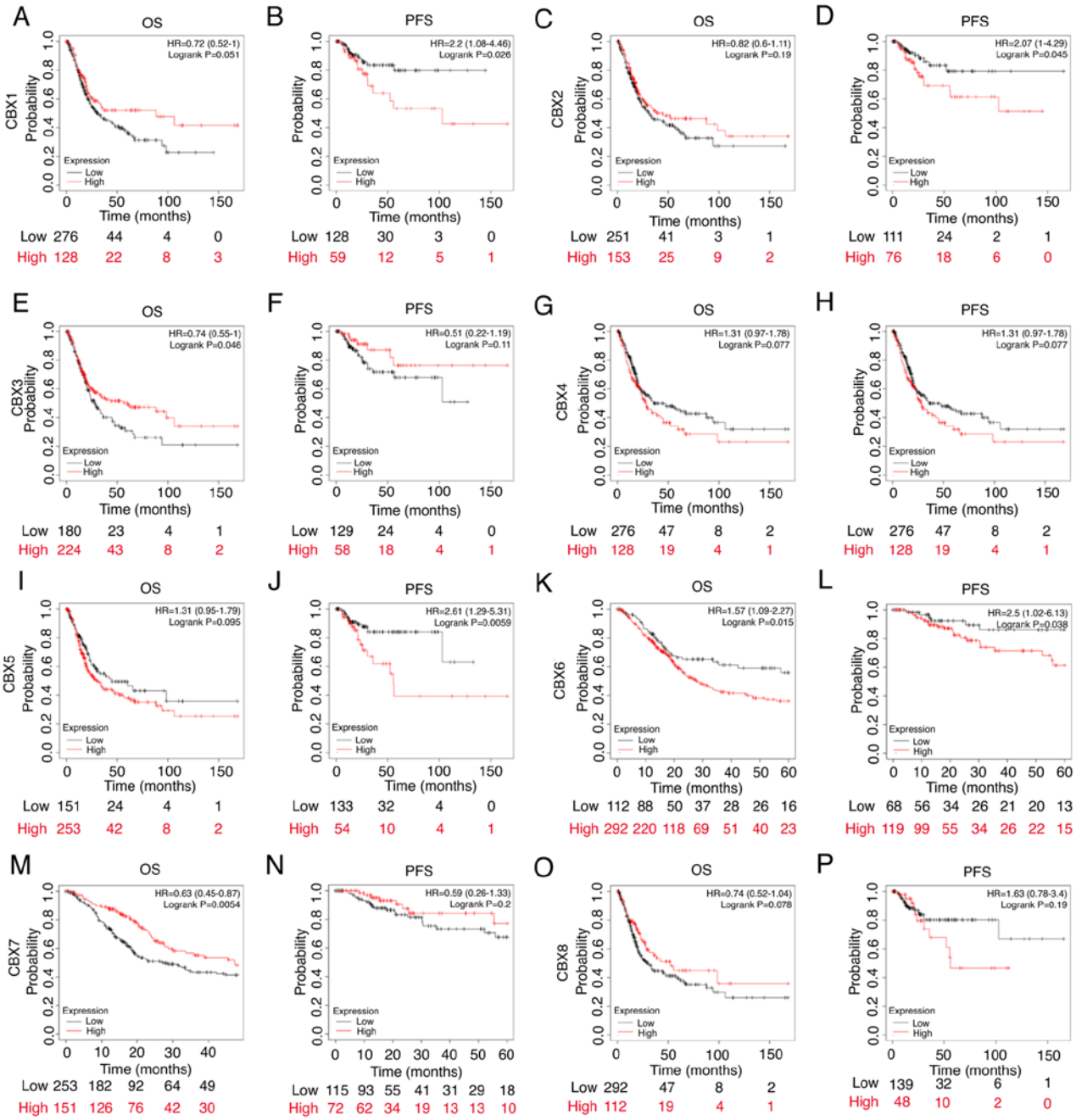

Figure 9. Prognostic value of CBXs in patients with BLCA in the OS and PFS curves generated by Kaplan-Meier plotter. (A) There was no significant difference in OS between patients with high and low expression of CBX1. (B) Patients with high expression of CBX1 had poorer PFS. (C) There was no significant difference in OS between patients with high and low expression of CBX2. (D) Patients with high expression of CBX2 had poorer PFS. (E) Patients with BLCA exhibiting low CBX3 expression had poorer OS. (F) There was no significant difference in PFS between patients with high and low expression of CBX3. (G) There was no significant difference in OS between patients with high and low expression of CBX4. (H) There was no significant difference in PFS between patients with high and low expression of CBX4. (I) There was no significant difference in OS between patients with high and low expression of CBX5. (J) Patients with high expression of CBX5 had poorer PFS. (K) Patients with high expression of CBX6 had poorer OS. (L) Patients with high expression of CBX6 had poorer PFS. (M) Patients with BLCA exhibiting low CBX7 expression had poorer OS. (N) There was no significant difference in PFS between patients with high and low expression of CBX7. (O) There was no significant difference in OS between patients with high and low expression of CBX8. (P) There was no significant difference in PFS between patients with high and low expression of CBX8. The significance was computed using the Cox-Mantel test to compare two cohorts. BLCA, bladder cancer; CBX, chromobox; OS, overall survival; PFS, progression-free survival.

A number of studies have demonstrated that CBX8 participates in different types of cancer. Zhang *et al* (66) reported that CBX8 overexpression promoted hepatocellular carcinoma progression by upregulating the AKT/β-catenin pathway. Additionally, Zeng *et al* (67) showed that CBX8 accelerated BLCA progression by downregulating PRDM1, thus promoting c-FOS expression. The present study found that CBX8 was significantly increased in BLCA tissues, and its overexpression was related to the tumor stage of patients, suggesting it exerts a tumorigenic role in BLCA.

CBX family members participate in chromosomal modification, transcriptional repression, cell differentiation and senescence (8). Previous studies have confirmed that CBXs are associated with a number of tumors (10,37,41,47). Herein, the

Table II. Cox proportional hazard model of CBXs and six tumor-infiltrating immune cells in BLCA performed using TIMER.

Variables	Coefficient	HR	HR 95%CI_l	95%CI_u	P-value
B_cell	-2.589	0.075	0.004	1.575	0.095
CD8_T cell	1.139	3.124	0.199	48.947	0.417
CD4_T cell	-0.446	0.640	0.016	26.018	0.813
Macrophage	5.128	168.639	18.450	1541.408	<0.001[a]
Neutrophil	-4.449	0.012	0.000	1.659	0.078
Dendritic	0.046	1.047	0.224	4.895	0.953
CBX1	-0.246	0.782	0.582	1.050	0.102
CBX2	0.053	1.054	0.899	1.236	0.516
CXB3	-0.111	0.895	0.613	1.306	0.564
CBX4	0.225	1.252	0.915	1.714	0.160
CBX5	0.177	1.194	0.956	1.491	0.118
CBX6	0.201	1.223	1.019	1.467	0.031[b]
CBX7	-0.346	0.708	0.569	0.881	0.002[c]
CBX8	-0.216	0.806	0.589	1.102	0.177

[a]P<0.001, [b]P<0.05, [c]P<0.01. HR, hazard rate; BLCA, bladder cancer; CBX, chromobox; TIMER, Tumor Immune Estimation Resource; CI_u, upper limit of 95% confidence interval; CI_l, lower limit of 95% confidence interval.

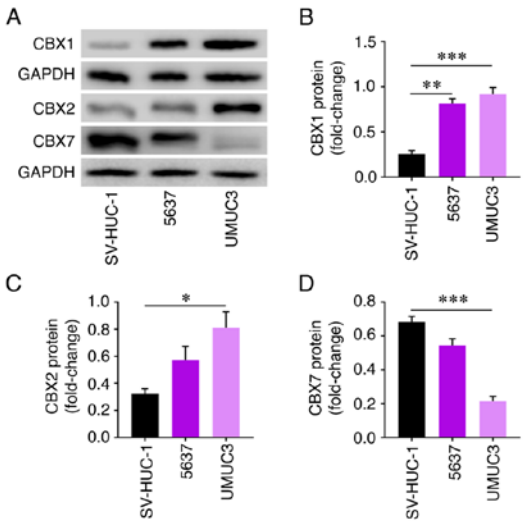

Figure 10. Protein expression levels of CBXs with prognostic value in different cell types. (A) Protein expression levels of CBX1, CBX2 and CBX7 in different cell types, and the bottom GAPDH blot is for both CBX2 and CX7. Semi-quantification of (B) CBX1, (C) CBX2 and (D) CBX7 protein expression levels determined by densitometry normalized to GAPDH. The difference between groups was evaluated by one-way ANOVA followed by the LSD post hoc multiple comparisons test. Data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. CBX, chromobox.

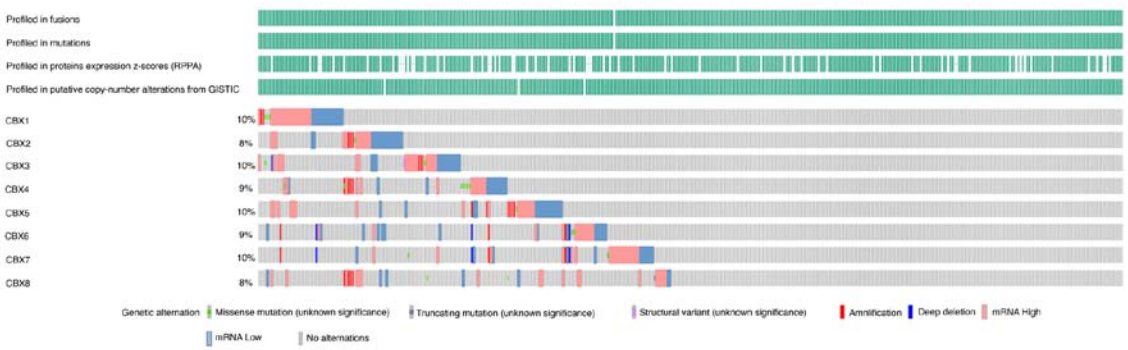

Figure 11. CBX gene mutation analyses in BLCA performed using cBioPortal. Summary of alterations in differentially expressed CBXs in BLCA. CBXs were altered in 194/407 (48%) samples from patients with BLCA. BLCA, bladder cancer; CBX, chromobox.

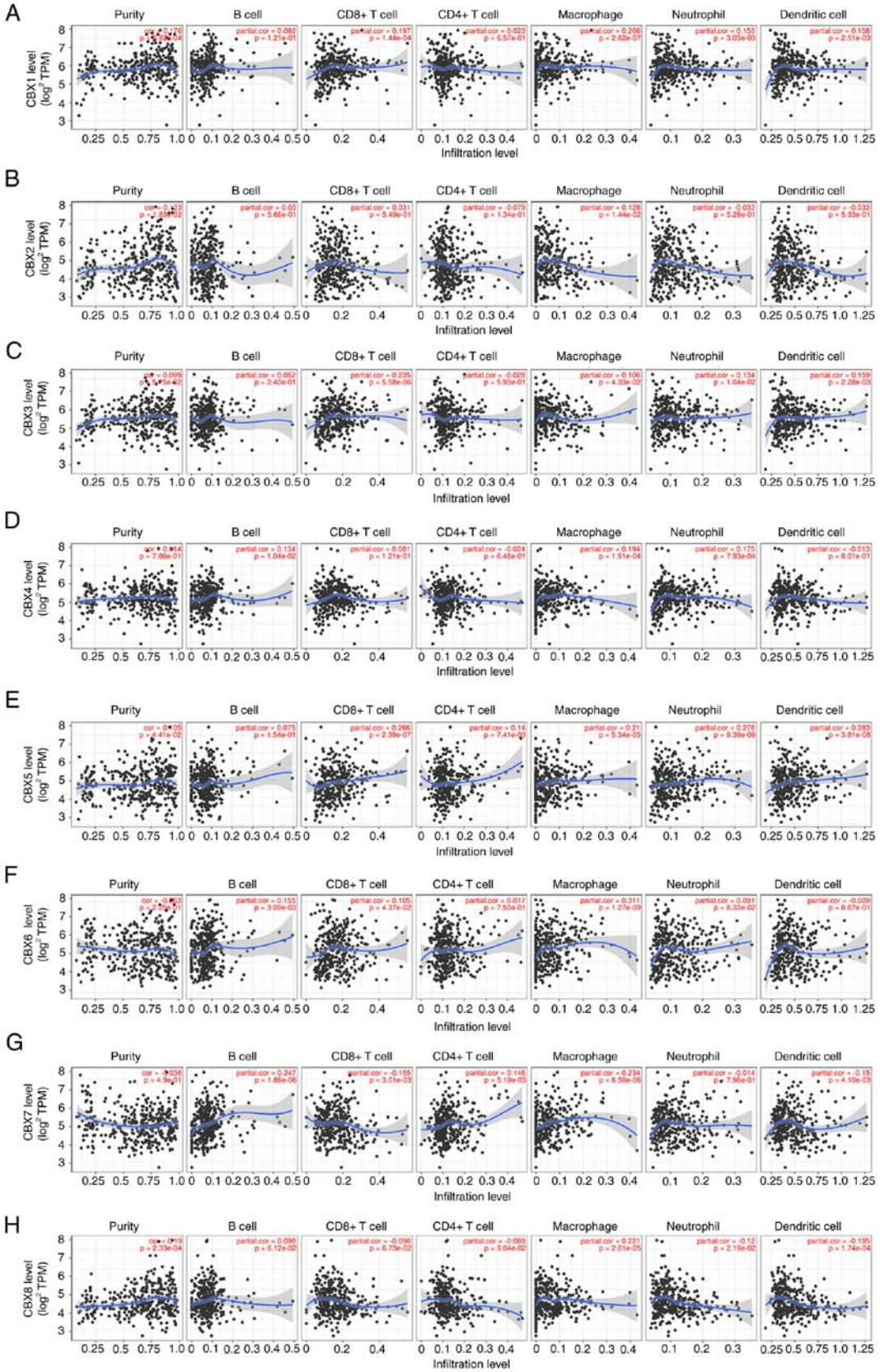

Figure 12. Correlation between differentially expressed CBXs and infiltration of immune cells using TIMER. The expression of (A) CBX1 was positively related to tumor purity and the infiltration of $CD8^+$ T cells, macrophages, neutrophils and dendritic cells; (B) CBX2 expression was positively related to tumor purity and the infiltration of macrophages; (C) CBX3 expression was positively related to the infiltration of $CD8^+$ T cells, macrophages, neutrophils and dendritic cells; (D) CBX4 expression was positively related to the infiltration of B cells, macrophages and neutrophils; (E) CBX5 expression was positively related to tumor purity and the infiltration of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells; (F) CBX6 expression was positively related to the infiltration of B cells, $CD8^+$ T cells and macrophages; (G) CBX7 expression was positively related to the infiltration of B cells, $CD4^+$ T cells, macrophages, and negatively associated with the infiltration of $CD8^+$ T cells and dendritic cells; (H) CBX8 expression was positively associated with tumor purity, the infiltration of macrophages and negatively associated with the infiltration of neutrophils and dendritic cells. TPM, transcripts per million; CBX, chromobox; TIMER, Tumor Immune Estimation Resource.

present study performed GO functional and KEGG pathway enrichment analyses for CBX family members and their related genes. The results revealed that CBX and their related genes were enriched in the 'mitotic cell cycle', 'RNA splicing' and 'cell division'. These results suggested that CBX might affect the pathogenesis of BLCA by participating in these biological processes. Previous studies have indicated that the infiltration of immune cells into tumors can affect the efficacy of immunotherapy and thus tumor progression in patients (68-71). The present study showed that the expression of CBXs was significantly associated with the infiltration of immune cells, including dendritic cells, neutrophils, macrophages, B cells, $CD4^+$ T cells and $CD8^+$ T cells. These results suggested that CBXs might affect the immune infiltration status of patients with BLCA. In summary, CBX1, CBX2, CBX3, CBX4 and CBX8 might promote BLCA progression, whereas CBX6 and CBX7 might exert a suppressive role. Although further investigations are needed to verify the results, the present study provides a theoretical basis for discovering new targets and prognostic markers for BLCA therapy.

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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

LBZ, BF and RFC designed the study and wrote the article. XQL, XM, LML, MCJ, KHW, YFL, QQZ and CY performed the literature search and data analysis. LBZ and XM confirm the authenticity of all the raw data. All authors 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.

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