Original Article Identification of a tumor microenvironment-associated prognostic gene signature in bladder cancer by integrated bioinformatic analysis

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Received October 8, 2020; Accepted November 30, 2020; Epub May 15, 2021; Published May 30, 2021

Abstract: Bladder cancer is a common malignancy in the urinary system. Stromal and immune cells in tumor microenvironments, including those in the bladder cancer microenvironment, can serve as prognostic markers. However, the complex processes of bladder cancer necessitate large-scale evaluation to better understand the underlying mechanisms and identify biomarkers for diagnosis and treatment. We used the Estimation of STromal and Immune cells in MAlignant Tumors using Expression data algorithm to assess the association between stromal and immune cell-related genes and overall survival of patients with bladder cancer. We also identified and evaluated differentially expressed genes between cancer and non-cancer tissues from The Cancer Genome Atlas. Patients were categorized into different prognosis groups according to their stromal/immune scores based on differential gene expression. In addition, the prognostic value of the differentially expressed genes was assessed in a separate validation cohort using the Gene Expression Omnibus microarray dataset GSE13507, which identified nine genes (TNC, CALD1, PALLD, TAGLN, TGFB111, HSPB6, RASL12, CPXM2, and CYR61) associated with overall survival. Multivariate regression analysis showed that three genes (TNC, CALD1, and PALLD) were possible independent prognostic markers for patients with bladder cancer. Multiple gene set enrichment analysis of individual genes showed strong correlations with stromal and immune interactions, indicating that these nine genes may be related to carcinogenesis, invasion, and metastasis of bladder cancer. These findings provide useful insight into the molecular mechanisms of bladder cancer development, and suggest candidate biomarkers for prognosis and treatment.

Keywords: TCGA, GEO, tumor microenvironment, ESTIMATE, overall survival, bladder cancer

Introduction

Bladder cancer (BC) is a common malignancy in the human urinary system. It is estimated that approximately 81,400 new cases of BC and 17,980 deaths occurred in the United States in 2020 [1]. Over the past 30 years, despite much progress in therapeutic options for BC, there has been no substantial improvement in the number of BC cases diagnosed and treated, or in the five-year survival rate [2]. Precisely identifying BC patients at high risk of recurrence or metastasis, or with worse clinical outcomes, could benefit treatment decisions. Several clinical characteristics, such as tumor size, muscularis propria invasion, and metastasis, have been recognized as independent prognostic factors in BC [3, 4]. However, these clinical data are not specific and cannot predict an individual patient survival. More accurate risk models are urgently needed to screen high-risk BC patients who may benefit from personalized adjuvant therapies.

Accumulating evidence suggests that the tumor microenvironment (TME) significantly affects not only cancer progression but also treatment responses and clinical outcomes [5, 6]. While the TME presents antineoplastic potential at the early stages of tumorigenesis, malignancies can tolerate this suppressive effect and transform the TME into one conferring pro-

malignancy functions [7]. In addition to cancer cells, the TME contains the surrounding vascular network, extracellular matrix, fibroblasts, and immune infiltrates [8]. For example, cancer-associated fibroblasts, considered as one of the most dominant components in the TME, were shown to facilitate the proliferation of cancer stem cells based on CXC-chemokine ligand 12-CXC-chemokine receptor 4 [9]. CAF-secreted cytokines, such as macrophage colonystimulating factor 1 and IL-6 promote monocyte recruitment and encourage the differentiation of tumor-associated macrophages in TME [10]. Though the complex interaction of cells in TME is not fully characterized, the molecular profiles of stromal components are of great value in serving as prognostic biomarkers [11]. TMEassociated prognostic multi-gene signatures have shown potential value in clinical outcome prediction and treatment guidance in lung cancer [12], glioma [13], and ovarian cancer [14], but no research has been reported in BC.

In the present study, we investigated the association of clinical parameters of BC patients with the stromal and immune scores calculated by Estimation of STromal and Immune cells in MAlignant Tumors using Expression data (ES-TIMATE) algorithm [15]. Through integrated bioinformatic analyses, we obtained a list of TMErelated genes and several independent prognostic factors. A nine-gene prognostic signature for BC was then established and validated to be clinically significant.

Materials and methods

Data acquisition

The expression profile of BC samples and their corresponding clinical information were downloaded from The Cancer Genome Atlas (TCGA) (TCGA-BLCA, https://portal.gdc.cancer.gov/repository) [16] and Gene Expression Omnibus (GEO) (GSE13507, https://www.ncbi.nlm.nih. gov/geo) databases. There were 408 BC samples in TCGA and 188 BC samples in GSE13507. The ESTIMATE algorithm was used for stromal and immune scoring, which divided the patients into high-score and low-score groups according to the median.

Differentially expressed genes (DEGs) and functional analysis

DEGs between the high-score and low-score groups were identified using the "limma" pack-

age v3.11 (http://www.bioconductor.org/packages/release/bioc/html/limma.html) in R software v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). The screening criteria of DEGs was |log, fold change| value > 1.5 and adjusted-P value < 0.05. Heatmap, plotted by "pheatmap" package v1.0.12 (https:// //cran.r-project.org/web/packages/pheatmap/) was used to display the DEGs. The protein-protein interaction (PPI) network was constructed using STRING database (https:// string-db.org/). Functional enrichment analysis of selected DEGs was based on Gene Ontology (GO) terms, including biologic processes, molecular functions, and cellular components, generated in the R packages of clusterProfiler v3.11 and org.Hs.eg.db v3.11 (http://www.bioconductor.org/).

Survival analysis and construction of the risk model

The prognostic values of DEGs were evaluated by the Kaplan-Meier method and log-rank test, and were shown as a survival curve using the R package of "survival" v3.1-12 (https:// cran.r-project.org/web/packages/survival/ index.html). Univariate and multivariate Cox regression models were used to identify independent prognostic risk factors. For the nine prognostically-significant genes obtained from TCGA and GEO, we conducted univariate Cox regression analysis to obtain regression coefficients. The risk score was calculated by the sum of gene expression levels multiplied by corresponding regression coefficients. We divided all samples into high- and low-risk groups based on the median value of the risk score.

Prediction of immunotherapy and chemotherapeutic response

We used subclass mapping to compare the expression profiles of the high- and low-risk groups with another published dataset, which contains 47 melanoma patients who received immunotherapy [17]. On the basis of the Genomics of Drug Sensitivity in Cancer, we predicted the chemotherapeutic response of each sample for four commonly used chemotherapy drugs in BC (cisplatin, bleomycin, mitomycin C and gemcitabine) using R package "pRRophetic" (https://github.com/paulgeeleher/pRRophetic2). Ridge regression was used to estimate the half-maximal inhibitory concentration (IC50).



Figure 1. Workflow chart.

Gene set enrichment analysis (GSEA)

The GSEA [18] analysis was conducted using GSEA v4.0.1 (http://software.broadinstitute. org/gsea/downloads.jsp). The referenced gene sets in GSEA were "c2.cp.kegg.v7.1.symbols. gmt" and "c5.all.v7.1.symbols.gmt". In this study, "permutations = 1000" was set to generate a null distribution for enrichment score and "FDR < 0.05" was applied as the threshold for screening. The other parameters for GSEA were set as defaults.

Statistical analysis

A comparison of the estimated IC50 of the high- and low-risk groups was performed using the Wilcoxon test. The Kaplan-Meier plotter was used to analyze the overall survival in TCGA and GEO, and the statistical differences between different groups were evaluated by log-rank test. Multivariate survival analysis was performed using Cox regression models. Hazard ratios were obtained with respective 95% confidence intervals. A *P* value < 0.05 was considered significant. The statistical tests were 2-sided and conducted using R software (version 3.5.3).

Results

Stromal and immune scores are associated with clinical traits of BC patients

The workflow chart is shown in Figure 1. Initially, the expression profile (FPKM) of TCGA-BLCA cohort containing 408 BC patients was downloaded to calculate stromal and immune scores. It was found that papillary carcinoma and nonpapillary carcinoma, two distinct pathologic types of BC, possessed significantly different stromal and immune scores. Compared with papillary BC, non-papillary BC had higher stromal (P < 0.001, Figure **2A**) and immune scores (P <0.001, Figure 2B). Low-grade and high-grade BC displayed significant differences in stromal (Figure 2C, P < 0.001) and immune scores (Figure 2D, P <

0.001), in which high-grade BC was associated with higher scores. Female patients had higher stromal (**Figure 2E**, P = 0.032) and immune scores (**Figure 2F**, P = 0.036) than male patients. This result is consistent with the fact that female patients with higher-grade BC usually had worse clinical outcomes than male patients [2]. These results revealed that patients with different BC subtypes, tumor grades, and genders had distinct TMEs.

Differentially expressed genes between high and low stromal/immune scores

The samples in TCGA-BLCA were divided into two groups of high and low stromal/immune scores according to the median value. A total of 1519 upregulated DEGs (\log_2 fold change > 1.5, *P* < 0.05) and 1371 downregulated DEGs (\log_2 fold change < -1.5, *P* < 0.05) were identified between the high and low stromal score groups (**Figure 3A**). Between the high and low immune score groups, 457 upregulated DEGs and 308 downregulated DEGs were identified (**Figure 3B**). When intersected, a total of 1125 shared upregulated DEGs (**Figure 3C**) and 209 (**Figure 3D**) shared downregulated DEGs were obtained. GO enrichment analysis of these



Figure 2. Analysis of bladder cancer (BLCA) stromal and immune scores by subtype and gender. A, B. Stromal (1) and immune (2) scores according to BLCA subtype (N = 403). C, D. Stromal (1) and (2) immune scores associated with BLCA grade (N = 405). E, F. Stromal (1) (P = 0.032) and (2) immune scores (P = 0.036) associated with patient gender (N = 408).

DEGs demonstrated that the top five enriched pathways were T cell activation, extracellular matrix organization, cytokine activity, leukocyte cell-cell adhesion, and leukocyte migration (**Figure 3E**).

PPI network of DEGs with prognostic value

Kaplan-Meier survival analysis revealed that 204 of the 1125 upregulated DEGs and 70 of the 209 downregulated DEGs were significantly associated with overall survival (data not shown). Using these 274 prognostic DEGs, a

PPI network with 92 nodes and 190 edges was constructed in the STRING database (**Figure 3F**). The central nodes (key genes) were as follows: *FN1*, *ITGB3*, *GAS6*, *TGFB3*, *TNC*, *VCAN*, *CYR61*, *IGF1*, *LCK*, *MFGE8*, *BDKRB2*, *CHRM2*, and *CXCL12* (**Figure 3G**). Survival curves of these key genes are displayed in <u>Supplementary Figure 1</u>.

Validation of prognostic DEGs

GSE13507 datasets in the GEO database were analyzed to verify the prognostic value of these





Figure 3. Comparison of bladder cancer stromal and immune scores with gene expression profiles. The heatmap was drawn using the average line and Pearson distance measurement methods. The differential gene matrix from the heatmap was corrected using the Wilcoxon test method. The more highly expressed genes in bladder cancer are shown in red, and the less highly expressed genes are shown in green. Genes with the same expression level between groups are indicated in black. A. Differentially expressed gene (DEG) heatmap (log₂ fold change > 1.5, *P* < 0.05) comparing groups with high and low stromal scores. B. DEG heatmap comparing groups with high and low immune scores (log₂ fold change > 1.5, *P* < 0.05). C. Venn plot showing upregulated DEGs along with stromal and immune scores related to biologic process, cell component, molecular function, top-10 GO pathway clusters, and the pseudo-discovery rate (*P* < 0.05). F. Protein-protein interaction (PPI) network of prognostic DEGs. G. Hub genes in the PPI network.

DEGs. Of the 161 prognostic genes identified using TCGA-BLCA, nine genes (TNC, CALD1, PALLD, TAGLN, TGFB111, HSPB6, RASL12, CPXM2, and CYR61) were validated to be of prognostic value in GSE13507 (Figure 4). The expression levels of these nine genes in BC and adjacent normal tissues are shown in Supplementary Figure 2. Univariate and multivariate analyses of TCGA-BLCA also revealed that TNC (Supplementary Table 1), CALD1 (Supplementary Table 2), and PALLD (Supplementary Table 3) emerged as independent prognostic markers of BC patients. We then conducted the GSEA to explore the biological processes in which these nine genes potentially participate. It was shown that these nine key genes were mainly involved in chemokine signaling, T cell receptor signaling, focal adhesion, extracellular matrix receptor interaction, and other immune and matrix-related pathways (Supplementary Figure 3). The results of GSEA further indicated that the nine key DEGs with prognostic value were TME-related genes, which is also in accordance with previously published literature [19-33].

Construction and validation of the nine-gene prognostic signature

The shared nine prognostic TME-related DEGs obtained from TCGA-BLCA and GSE13507 were then included in a multivariate Cox regression to acquire coefficients. A TME-associated ninegene signature was developed: risk score = $0.0029 \times \text{TNC} + (-0.0120) \times \text{CALD1} + 0.0126 \times \text{PALLD} + (-0.0011) \times \text{CYR61} + (-0.0031) \times \text{HSPB6} + 0.0384 \times \text{CPXM2} + 0.0127 \times \text{RASL12} + 0.0615 \times \text{TGFB1I1} + (-0.0017) \times \text{TAGLN}$. BC patients were divided into high-risk and low-risk groups according to the median risk score. Survival analysis of TCGA-BLCA showed that compared with the high-risk group, BC patients in the low-risk group had significantly longer overall survival (**Figure 5A**) and longer progression-free survival (**Figure 5B**). This nine-gene prognostic signature was then validated in GSE13507, and a satisfactory predictive significance was achieved (**Figure 5C** and **5D**).

Prediction of immunotherapeutic and chemotherapeutic response

The expression profiles of the high- and lowrisk groups were compared with those in a published dataset containing 47 melanoma patients who responded to immunotherapy by subclass mapping [34] to evaluate immunotherapeutic and chemotherapeutic response of BC patients. The high-risk group showed significantly greater sensitivity to anti-PD-1 treatment (Bonferroni correction, P = 0.048, **Figure 6A**).

In addition, considering that chemotherapy is the most common treatment of BC, we evaluated the response of the high- and low-risk groups to four common chemotherapy drugs. There was no significant difference in IC50 estimated by cisplatin between the high- and lowrisk groups (P = 0.973, Figure 6B), Significant differences were achieved in IC50 estimated by bleomycin (Figure 6C), mitomycin C (Figure 6D) and gemcitabine (Figure 6E). Compared with the low-risk group, bleomycin, mitomycin C, and gemcitabine showed a greater sensitivity of therapeutic response to BC patients in the high-risk group. Enrichment analyses of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO were then conducted to reveal the underlying mechanisms. The gene expression profiles of BC patients in the high-risk group were mainly enriched in various TMEassociated processes, such as extracellular matrix receptor interaction, focal adhesion pathway, migration of macrophages, and composition of extracellular matrix (Figure 7).

Discussion

The incidence and mortality of bladder cancer (BC), a common and complex disease, in-





Figure 4. Validation of overall survival associated with DEGs extracted from The Cancer Genome Atlas using Gene Expression Omnibus data. Kaplan-Meier survival curves between the groups with highly expressed genes (red line) and lowly expressed genes (blue line) (*P* < 0.05 in the log-rank test). OS, overall survival in years.



Figure 5. Survival analysis between high- and low-risk groups in The Cancer Genome Atlas and GSE13507. Kaplan-Meier survival curves show the (A) overall survival (OS), (B) progression-free survival (PFS) and (E) relapse-free survival (RFS) in TCGA, together with (C) OS and (D) PFS in GSE13507.



Figure 6. Prediction of immunotherapy and chemotherapeutic response between high- and low-risk groups. (A) Prediction response of immune checkpoint inhibitors anti-CTLA4 and anti-PD1 between high- and low-risk groups. The high-risk group showed it was more sensitive to the anti-PD1 inhibitor (Bonferroni-corrected P= 0.048). Estimated IC50 for (B) cisplatin, (C) bleomycin, (D) mitomycin C and (E) gemcitabine.

creased by 2.5 and 1.6 times, respectively, between 1990 and 2013 [35]. There has been significant progress in understanding the association between overall survival and gene expression in BC, including experiments from animal tumor models, in vitro tumor cell lines, and patient tumor samples. However, the complexity of BC and the associated microenvironments necessitate broader analyses using larger cohorts. The present study integrated clinical information associated with BC from TCGA data with stromal and immune scores based on the ESTIMATE algorithm, revealing a set of nine DEGs (TNC, CALD1, PALLD, TAGLN, TGFB111, HSPB6, RASL12, CPXM2, and CYR61) in BC tumor microenvironments associated with poor prognosis for patients with BC. The prognostic significance of these genes was verified using an independent GEO cohort.

CALD1, PALLD, CYR61, TGFB1/1, and TAGLN are all widely expressed in smooth muscle cells, and either directly or indirectly participate in cancer cell growth, proliferation, and migration, as well as inhibit immune cells to help cancer cells invade the muscle tissue [36-43]. TNC encodes an extracellular matrix (ECM) glycoprotein that is highly expressed during organogenesis, and is associated with cell proliferation and migration, epithelial-mesenchymal transition, and parenchymal and mesenchymal interactions [44, 45]. HSPB6 is also highly

expressed in muscle tissues and may be the main mediator of muscle protection signal transduction in tumors invading the muscle layer, although its specific role in cancer is not clear [46]. CALD1 encodes an actin-regulating protein that is usually expressed in smooth muscle and non-muscle cells. CALD1 also plays a role in the actin cytoskeleton and participates in a variety of biologic functions associated with tumors, such as migration, invasion, and proliferation [47]. In patients who underwent colorectal cancer treatment, tumor cell growth was found to significantly decrease, and invasion and migration were inhibited with an increase in CALD1 expression levels [48]. In addition, CALD1 inhibits cell invasion by reducing ECM degradation activity [36], as cell invasion is mediated by podocytes that degrade the ECM by secreting matrix metalloproteinases, allowing cells to pass through the basement membrane and invade tissues, which are signs of cancer progression. Nevertheless, the specific immune function of CALD1 remains uncertain, and further research is needed to better understand its role in cancers. PALLD encodes an actin-related protein expressed in various cell types that is essential for establishing cell morphology and maintaining cytoskeletal organization. PALLD expression is absent or low in peripheral blood mononuclear cells and causes dendritic cells to differentiate into monocytes, whereas its expression is significantly upregu-





Figure 8. Cellular localization and biologic processes associated with the nine DEGs. *CALD1, PALLD, TAGLN, TGFB1/1, HSPB6, RASL12,* and *CPXM2* are mainly expressed in smooth muscle cells (SMCs). *TNC* and *CYR61* are mainly expressed in tumor cells or fibroblasts/myofibroblasts. The BLCA-related biologic processes that they may participate in are as follows: TNC: a, b, c, d; *CALD1*: e, f, g; *PALLD*: b; *CYR61* (*CCN1*): h, i, j, l; *TGFB1/1*: f, g, h, k; *TAGLN*: e, f, g; and *HSPB6, RASL12,* and *CPXM2*: unknown.

lated during cell proliferation. There is also evidence that *PALLD* promotes phagocytic cup extension and closure [37]. However, the precise mechanisms by which *PALLD* regulates innate immunity and other biological processes remain unclear. The function and molecular mechanisms of *RASL12* and *CPXM2* are not known. The recognized roles of these nine DEGs in the BC microenvironment are summarized in **Figure 8**.

The functions of these nine genes in BC should be confirmed clinically and experimentally. Multi-pathway GSEA of each of the nine genes showed that they are jointly involved in calcium, transforming growth factor-beta, mitogen-activated protein kinase, and JAK-STAT signaling, and contribute to cancer progression by regulating cell proliferation, differentiation, apoptosis, and immune regulation. Focal adhesion, ECM receptor interactions, cytokine-cytokine

receptor interactions, and other pathways directly related to tumor microenvironments were also implicated. The ECM is a highly dynamic structure that facilitates structural and biochemical signaling to regulate cell function; it is present in all tissues and is constantly undergoing controlled remodeling, and is further considered to be a driver of fibrosis. In addition, the ECM interacts with cells to regulate multiple functions, includingproliferation, migration, and differentiation of tumor cells. During cancer progression, epithelial cells undergo genetic alterations that, along with matrix changes such as ECM remodeling, disrupt homeostasis of the epithelium. For example, parallel tissues composed of matrix ECM fibrils are associated with tumorigenic responses [49-51]. Focal adhesions are the main hubs of cellular mechanical sensing and act as bridges between integrin-ECM and

the cytoskeleton. Recent studies have shown that cell proliferation and RhoA GTPase activity control focal adhesion formation through *YAP*, thereby anchoring the actin cytoskeleton to the cell membrane and determining cell shape, migration, and differentiation. Changes in the signals transmitted through focal adhesions of malignant cells are crucial for the spread of tumor cells [52-54].

Cytokine-cytokine receptor interactions were also prominent functions identified in the present study. Cytokines are multifunctional molecules that can regulate broad-spectrum biologic events related to inflammation, metabolism, cell growth, differentiation, morphogenesis, fibrogenesis, and/or homeostasis. Immune regulation determines the nature and type of immune response [55-57]. Therefore, these nine genes have their own unique functions in BC microenvironments and play important roles in common signaling pathways. Finally, we systematically analyzed the risk model of the nine-gene signature. The risk coefficients of TNC, PALLD, CPXM2, RASL12, and TGFB1/1 were positive, and the coefficients of CALD1, CYR61, HSPB6, and TAGLN were negative, revealing their different contributions to the risk score. Although the high expression of all nine genes in the signature showed a poor prognosis for BC, the risk score further helped us identify which genes (TNC, PALLD, CPXM2, RASL12 and TGFB111) have a greater association with poor prognosis for BC. In addition, the high-risk group showed sensitivity to anti-PD1 immunotherapy and to the conventional chemotherapy drugs bleomycin, mitomycin C, and gemcitabine, all of which help with the treatment and diagnosis of bladder cancer in the future.

Based on our in-depth analysis of BC microenvironment transcriptome data, we confirmed that *TNC*, *CALD*, *PALLD*, *CYR61*, *TGFB1I1*, *TAGLN*, *HSPB6*, *RASL12*, and *CPXM2* are biologic markers of poor prognosis for patients with BC. The relevance of these genes to BC prognosis was validated in independent BC cohorts. However, the biologic functions and molecular mechanisms associated with *HS*-*PB6*, *RASL12*, and *CPXM2* in BC microenvironments should be explored in future research.

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (2016-GXNSFAA380306), the self-generated project of the Guangxi Zhuang Autonomous Region Health Department (Z20170816), China.

Disclosure of conflict of interest

None.

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Supplementary Figure 1. Survival analysis of central nodes (key genes) in PPI network. Kaplan-Meier survival curves between the groups with highly expressed genes (red line) and low expressed genes (blue line) (P < 0.05 by the log-rank test). OS, overall survival in years.



Supplementary Figure 2. Expression levels of nine genes in BC and adjacent normal tissues using TCGA data. TNC, CALD1, PALLD, TAGLN, TGFB111, HSPB6, RASL12, CPXM2, and CYR61 are all highly expressed in adjacent normal tissues (P < 0.05).

Variable	Univariate analysis			Multivariate analysis			
	HR	HR. 95 CI	Р	HR	HR. 95 CI	Р	
Age	1.03	1.00-1.06	0.040	1.03	1.00-1.06	0.044	
Gender	0.63	0.36-1.10	0.106	0.68	0.38-1.22	0.200	
Stage	1.78	1.24-2.54	0.002	1.15	0.56-2.33	0.705	
Т	1.70	1.15-2.50	0.008	1.30	0.78-2.16	0.315	
Μ	2.12	0.76-5.88	0.150	1.24	0.38-4.03	0.717	
Ν	1.55	1.18-2.03	0.002	1.23	0.73-2.08	0.444	
TNC	1.27	1.11-1.46	0.001	1.20	1.04-1.39	0.011	

Supplementary Table 1. Univariate and multivariate analyses of *TNC* expression for overall survival among bladder cancer patients with covariate adjustment

HR: hazard ratio; 95 Cl: 95% confidence interval. T: tumor; M: metastasis; N: lymph node.

Supplementary Table 2. Univariate and multivariate
analyses of CALD1 expression for overall survival among
bladder cancer patients adjusted for covariates

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Variable	Univariate analysis			Multivariate analysis			
variable	HR	HR. 95 CI	Р	HR	HR. 95 CI	Р	
Age	1.03	1.00-1.06	0.040	1.03	1.00-1.05	0.091	
Gender	0.63	0.36-1.10	0.106	0.65	0.37-1.16	0.144	
Stage	1.78	1.24-2.54	0.002	1.10	0.53-2.30	0.788	
Т	1.70	1.15-2.50	0.008	1.37	0.81-2.31	0.235	
Μ	2.12	0.76-5.88	0.150	0.93	0.28-3.07	0.911	
Ν	1.55	1.18-2.03	0.002	1.30	0.77-2.22	0.322	
CALD1	1.36	1.12-1.66	0.002	1.30	1.02-1.55	0.030	

HR: hazard ratio; 95 Cl: 95% confidence interval. T: tumor; M: metastasis; N: lymph node.

Supplementary Table 3. Univariate and multivariate analyses of *PALLD* expression for overall survival among bladder cancer patients adjusted for covariates

Variable	Univariate analysis			Multivariate analysis		
	HR	HR. 95 CI	Р	HR	HR. 95 CI	Р
Age	1.03	1.00-1.06	0.040	1.030	1.00-1.06	0.081
Gender	0.63	0.36-1.10	0.106	0.630	0.35-1.11	0.110
Stage	1.78	1.24-2.54	0.002	1.170	0.57-2.40	0.660
Т	1.70	1.15-2.50	0.008	1.310	0.78-2.22	0.310
Μ	2.12	0.76-5.88	0.150	1.000	0.31-3.21	0.998
Ν	1.55	1.18-2.03	0.002	1.260	0.75-2.13	0.378
PALLD	1.44	1.14-1.83	0.002	1.320	1.03-1.70	0.026

HR: hazard ratio; 95 Cl: 95% confidence interval. T: tumor; M: metastasis; N: lymph node.



Supplementary Figure 3. Gene Set Enrichment Analysis of nine genes in BC. KEGG analysis between high and low expression groups of these nine genes. FDR < 0.05 was the screening threshold.