JAM3 is a potential risk biomarker for predicting prognosis and immune cell infiltration by regulating epithelial–mesenchymal transition in bladder cancer

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DOI: https://doi.org/10.17305/bb.2024.9979

Submitted: 29 October 2023/ Accepted: 09 February 2024/ Published online: 23 February 2024

Conflicts of interest: Authors declare no conflicts of interest.

Funding: This study was supported by Horizontal project of Heilongjiang Renxin Medical Assistance Foundation and Heilongjiang Charity Fund General Association. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability: The data generated in this study are publicly available in Gene Expression Omnibus (GEO) at GSE3167, GSE13507, GSE52519, GSE65635, GSE100926 and GSE120736, and in The Cancer Genome Atlas for bladder cancer data.
ABSTRACT

To identify novel biomarkers for predicting prognosis and immune function in bladder cancer (BC) patients, we combined weighted correlation network analysis (WGCNA) and least absolute shrinkage and selection operator (LASSO) regression analysis by using data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, finally screened the junctional adhesion molecule 3 (JAM3) as an independent risk factor in BC. High levels of JAM3 were linked to adverse clinical parameters, such as increased T and N stages. Additionally, a JAM3-based nomogram model accurately predicted 1-, 3- and 5-year survival rates of BC patients, indicating potential clinical utility. Functional enrichment analysis revealed that high JAM3 expression activated calcium signaling pathway, the ECM-receptor interaction and the PI3K-Akt signaling pathway, and was positively correlated with genes associated with epithelial–mesenchymal transition (EMT). Subsequently, we found that overexpression of JAM3 promoted the migration and invasion abilities in BC cells, regulating the expression levels of N-cadherin, matrix metallopeptidase 2 (MMP2) and Claudin-1 thereby promoting EMT levels. Additionally, we showed that JAM3 was negatively correlated with anti-tumor immune cells such as CD8+ T cells, while positively correlated with pro-tumor immune cells such as M2 macrophages, suggesting its involvement in immune cell infiltration. The immune checkpoint CD200 also showed a positive correlation with JAM3. Our findings revealed that elevated JAM3 levels are predictive of poor prognosis and immune cell infiltration in BC patients by regulating EMT process.

KEYWORDS: Junctional adhesion molecule 3 (JAM3), bladder cancer, epithelial–mesenchymal transition, prognostic biomarker, immune cell infiltration
INTRODUCTION

Bladder cancer (BC) is the most commonly diagnosed tumor in urinary system with the high mortality, causing societal burden worldwide [1,2]. Unfortunately, the mechanisms underlying BC initiation, proliferation and progression remain largely unknown. Known risk factors include age, gender, cigarettes, genetic factors and other environmental influences [3]. At present, the mainstay treatment for BC including surgery, chemotherapy and immune therapy have markedly improved patient outcomes. However, high recurrence rates, as well as the side effects or the limited effectiveness in advanced or metastatic stages of BC continue to result in significant mortality [4]. Accordingly, it is imperative to discover new methods or biomolecules with effective value on early detection or improvement of BC prognosis.

Based on whether the tumor cells invade the muscle layer of the bladder wall, BC can be classified into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). MIBC exhibits a more aggressive nature, correlates with a higher level of T-stage, and has more significant genetic heterogeneity, in which impaired DNA damage response and repair pathways are very common [5]. Additionally, studies have been done to use changed genes that are strongly linked to the development of BC to classify the disease into several molecular subtypes [6].

Immunotherapy has emerged as a pivotal treatment for advanced and metastatic BC. The behavior of BC cells, their response to treatment, and the prognosis of individuals with BC are all influenced by the immunological microenvironment, which has been the focus of research [7,8]. Note that the immune checkpoint inhibitors (ICIs) are the first-line treatment among immunotherapies, targeting expression levels of programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which are associated with immunotherapy response[9]. A recent study demonstrated that targeting AR to reduce PD-L1 expression can enhance the tumor-killing
abilities of NK cells [10]. However, the success rate of anti-PD1/PDL1 treatment in advanced BC patients is estimated to be around 20% [11]. Besides, it is reported that therapeutic responsiveness of ICIs treatment was closely associated with tumor microenvironment and tumor-infiltrating immune cells [12]. Nevertheless, the fact that immune cells can act as protective factors (anti-tumor immunity) or risk factors (pro-tumor immunity) in the tumor microenvironment makes them a "double-edged sword" [13]. For example, the roof plate-specific spondin (RSPO) family has been found to influence the development of BC by regulating the invasion of CD4 T cells and macrophages [14]. Still, investigating the tumor-infiltrating immune cell levels could help understand the mechanism of tumor immunity and predict the ICIs responses.

The occurrence of genetic alterations is believed to be closely linked to BC tumorigenesis [15]. Therefore, investigating the genetic alterations might offer opportunities to further understand biological changes in BC. Recent years, bioinformatics has been an important method in cancer research. One widely used approach is analyzing the expression of differentially expressed genes [16]. Another powerful method is the weighted gene co-expression network analysis (WGCNA), which can reveal patterns of gene expression and identify highly significant genes associated with specific traits [17]. Additionally, the Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis can be utilized to identify the most important combination of independent variables and regression coefficients for the most accurate predictive model [18].

JAM3, located on the 11q25 region of the human chromosome, is a member of the JAM family, which are cell-cell adhesion molecules of the immunoglobulin superfamily. JAM3 is expressed in various tissues and plays a crucial role in cell junctions, cell polarity, and motility [13-15]. It has been proposed that JAM3 participates in leukocyte-platelet interactions, as well as angiogenesis and brain development [17]. In the field of cancer
research, the expression of JAM3 is silenced by the gene methylation in colorectal cancer and esophageal cancer, showing a close relationship between gene functions of JAM3 and its relative methylation level [19]. It is important to note that the roles of JAM3 are reported different and controversial in multiple cancers. In leukemia, JAM3 maintains leukemia-initiating cell function through the LRP5/AKT/β-catenin/CCND1 signaling pathway and is associated with poor prognosis in leukemia[26]. While the methylation level of JAM3 is identified as an independent risk factor in esophageal cancer by activating the Wingless-related integration site (Wnt) signaling pathway, showing the tumor suppression function of JAM3[24]. However, the roles of JAM3 in BC are largely unknown at present.

The epithelial–mesenchymal transition (EMT) was considered as a classic molecular mechanism of tumor metastasis, with remarkable changes in expression levels of several crucial EMT-related proteins, including zinc finger E-box binding homeobox (ZEBs) proteins, Snails proteins, matrix metalloproteinases (MMPs) proteins, Claudin-1, Vimentin, Cadherin-1 (CDH1) and Cadherin-2 (CDH2) which gained a lot of attention on the treatment for multiple cancers[27]. While the involvement of JAM3 in EMT has been documented in gastric cancer [28], its regulation in breast cancer still lacks sufficient evidence.

In summary, we obtained 16 genes by combining differentially expressed genes, WGCNA and applied LASSO regression analysis. Then results of univariate and multivariate cox analysis of these 16 genes revealed that only JAM3 was an independent prognostic factor in BC. Previous studies have not been able to elucidate the specific role of JAM3 in breast cancer. Therefore, further research on the relationship between JAM3 and breast cancer cell behavior, particularly in relation to EMT, is necessary for a comprehensive understanding of its function in this disease. This understanding can contribute to prognostication, tumor progression, and guide treatment strategies to improve patient survival. Our findings indicate
that high levels of JAM3 are a significant prognostic indicator for predicting unfavorable outcomes, adverse clinical features, and reduced immune cell infiltration in breast cancer.

In this study, we identified JAM3 as an independent risk factor in BC, with further investigation to assess its prognostic value and perform preliminary functional exploration to indicate JAM3 regulates the EMT process in BC, thus providing a new marker for predicting the prognosis and immune functions of BC patients.

**MATERIALS AND METHODS**

**Data download and collation**

In this study, we acquired transcriptome data and clinical information for 394 BC cases and 87 normal cases from six Gene Expression Omnibus (GEO) cohorts (GSE3167, GSE13507, GSE52519, GSE65635, GSE100926 and GSE120736) available on the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Clinical traits of the samples are displayed in Figure S4. Meanwhile, transcriptome data, related clinical information and the DNA methylation data were downloaded from the Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga/) for 431 cases (412 BC cases and 19 normal cases). Then we used R package sva (version 3.44.0) to batch the correction of these six GEO cohorts and obtained a vast GEO cohort for further investigation.

**Identification of bladder cancer (BC) related genes in the vast Gene Expression Omnibus (GEO) cohort**

Firstly, we standardized all transcriptomics data from vast GEO cohort by applying log2(x+1). Then we applied the edge R package with FDR <0.05 and |log2FC| ≥1 to identify differentially expressed genes (DEGs) of transcriptomics in both TCGA and GEO cohort. Meanwhile, we used the WGCNA for identifying BC related genes preliminarily by applying R package WGCNA (version 1.71). During the WGCNA analysis, we set power value as
seven to complete the process and set Module Membership to 0.8 and Gene significance to 0.2 to identify BC related genes in the most significant module. After that, we intersected DEGs with BC related genes and applied the LASSO cox analysis to further filtrate these genes for identifying BC related genes.

**Identification of prognostic BC related genes**

To identify those BC related genes with prognostic value for further investigation, we applied univariate and multivariate Cox regression analyses and Kaplan-Meier analysis by using data from TCGA cohort.

**Gene biological function and immune function analysis**

We applied Gene Set Variation Analysis (GSVA), Disease Ontology (DO), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) for gene functional or pathway enrichment analysis. Then, R package limma (version 3.52.4) and algorithm of cibersort were used to assess the immune infiltration levels of BC cases.

**Protein-protein interaction (PPI) network**

We generated a protein-protein interaction (PPI) network for correlated expressed genes by using STRING (https://string-db.org/) and Cytoscape software.

**Cell culture**

In this study, we obtained human normal bladder cell line SVHUC-1, and human bladder cancer cell lines T24, RT112 and UMUC3 from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The SVHUC, T24 and RT112 cells were cultured in the RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, while UMUC3 cell was cultured in the DMEM (Gibco)
supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Both cell lines were cultured at 37°C and in 5% CO2.

**Transfection assay**

We obtained the overexpression plasmid of JAM3 and an empty plasmid from GeneCopoeia (Guangzhou, China). Firstly, we planted BC cells into 6-well plates and waited for cell density to reach 80%-90%. Then, we changed the complete culture medium into 1.5ml Opti-MEM (Gibco) for each well, 2 hours before transfection. Two groups were set up for transfection: negative control (NC) group and overexpression (OE) group. For the OE group, 10ul of the JAM3 overexpression plasmid was added to 240ul of Opti-MEM for each well. To this, 5ul of lipo2000 (Thermo, USA) was added to 245ul of Opti-MEM and allowed to sit at room temperature for 5 minutes. The plasmid and lipo2000 solutions were then mixed and allowed to sit for an additional 10 minutes. The resulting transfection solution (500ul) was added to each well, resulting in a 2ml volume transfection system. The NC group followed the same protocol; however, the overexpression plasmid was replaced with an empty plasmid. After 8 hours of transfection, the culture medium was changed to 2ml of RPMI-1640 supplemented with 10% serum (DMEM was used as the basic medium for UMUC3 cells). Transfection efficacy was confirmed by examining the fluorescence percentage and wells with a minimum of 80% fluorescence were selected for further investigation.

**Wound healing assay**

We planted OE cells and NC cells into 6-well plates and allowed them to reach full cell density. Then, we utilized a 200ul pipette tip to create lines and generate wounds. After washing with a PBS solution three times, we supplemented each well with 2ml of RPMI-1640 basic medium (DMEM for UMUC3 cells) and captured images at 0h, 24h, and 48h for analysis of the wound healing assay results.
Transwell assay

This section was divided into migration and invasion assay. For migration assay, the transinfected cells were incubated in serum-free medium for 12 hours and then adjusted to a density of $1 \times 10^6$ cells/ml. Next, 200μl serum-free cell suspension was added to the transwell chambers (Corning, NO.3422), which were fit into the wells of 24-well plates. The wells of the plates also each contained 500μl of basic medium supplemented with 10% FBS. After 24 hours of incubation, cells on the upper membrane were removed with cotton wool, whereas cells adhering to the lower surface were fixed in methanol for 30 minutes and then stained with 0.1% crystal violet for 20 minutes. After natural air drying, migrating cells on the lower surface of the membrane were then counted under an optical microscope at 200× magnification. For invasion assay, the same protocol was followed except that the transwell chambers were pre-treated with Matrigel extracellular matrices (Corning, USA). The dilution ratio for the extracellular matrices was 1:8.

Western blot assay

Before we processed the western blot assay, we used BCA protein assay kit (Beyotime, Beijing) to determine the loading sample. Twenty micrograms of total protein were separated by SDS-PAGE on 12% gradient polyacrylamide gels. Gels were electroblotted onto nitrocellulose membranes. For immunodetection, blots were blocked with 1% blocking reagent in 0.05% Tween 20-PBS for 1 h and incubated with primary antibody overnight at 4°C diluted in blocking buffer. The dilutions used in Western blots were anti-beta-Tublin (1:5000), anti-N-cadherin (1:2000), anti-Claudin-1 (1:1000), anti-MMP2 (1:1000) and anti-JAM3 (1:5000). The anti-JAM3 was purchased from Abmart (Shanghai, China), while the remaining primary antibodies were purchased from Abcam. Blots were then washed in 0.05% Tween 20-PBS and incubated with goat anti-rabbit (1:25000) (Abclonal, China) peroxidase
labeled antibody in a blocking buffer for 1h. An enhanced chemo luminescent system was applied. Scanning densitometry was performed with scan analysis software.

**Ethical statement**

TCGA database and GEO database are public databases, and there is no ethical conflict. Meanwhile no ethics statement was required from the institutional review board for the use of these prostate cancer cell lines.

**Statistical analysis**

We employed one-way ANOVA and t test for comparison between groups, and the comparison of two or more constituent ratios was used by chi-square test. Correlation analysis, heatmaps, receiver operating characteristic (ROC) curves, box plots and violin plots were completed by R software (version 4.2.1). Data of pan-cancers was downloaded from TIMER 2.0 database (http://timer.comp-genomics.org/). Statistics of relative protein expressions was completed by using ImageJ and Graphpad Prism softwares (version 10.0.2). R software was used for additional statistical analyses. A significance level of P < 0.05 was deemed appropriate for this study.

**RESULTS**

172 differentially expressed genes (DEGs) were screened from the vast GEO cohort

At first, we obtained a vast GEO cohort containing the expression of 9972 genes by merging six GEO datasets (GSE3167, GSE13507, GSE52519, GSE65635, GSE100926 and GSE120736), which included 87 normal samples as controls and 394 BC samples. Using an FDR threshold of less than 0.05 and a |log2 (FC)| of at least 1, we identified 172 DEGs (24 up-regulated and 148 down-regulated) between the BC samples and controls. All DEGs are shown by the volcano map, and the most relevant 100 DEGs are depicted in the heatmap (Figure S1A and B). Our GO and KEGG enrichment analysis revealed that these DEGs are
primarily associated with extracellular matrix functions and pathways (Figure S1C and D) (qvalue < 0.05). Furthermore, our DO analysis highlighted a significant enrichment in urinary system cancer (qvalue < 0.05), further confirming the strong connection between these DEGs and BC (Figure S1E).

Furthermore, we conducted GSEA to investigate the pathways enriched in the normal and BC tissues. We found that pathways related to cell adhesion molecules, cytokine-cytokine receptor interaction, and focal adhesion were active in normal tissues. In contrast, pathways commonly associated with tumor development, such as cell cycle, DNA replication, and bladder cancer, were predominantly active in BC tissues (Figure S1F and G). These findings preliminarily showed the strong relationship between DEGs and BC.

58 BC related genes were obtained by applying weighted correlation network analysis (WGCNA)

In order to explore the genes with similar expression patterns among the 9972 genes of BC, we adopted the WGCNA analysis to construct the gene co-expression network. First, we evaluated the sample clustering dendrogram of 481 samples (Figure S2A). Next, we established the ideal soft thresholding power which mainly influenced the scale independence and mean connectivity of gene co-expression modules. We chose seven as the power value based on the analysis of the scale-free index and mean connectivity for various soft-threshold powers (Figure S2B), as well as the selected power value could construct a scale-free network well ($R^2 = 0.88$) (Supplementary Figure 2C). Subsequently, these 9972 genes were divided into four different co-expression modules, represented by different colors (Figure S2D and E). Additionally, we created a heatmap to illustrate the correlation analysis findings between each module and clinical traits (Figure 1A), and we discovered that the turquoise module had the most significant ($r = -0.45, P = 5e-25$) association with the BC. Similarly, the turquoise module was the most important in determining whether the sample belonged to BC or not.
Finally, we obtained 58 related genes from the turquoise module when we set ‘module membership’ to 0.8 and ‘gene significance’ to 0.2 (Figure 1C). In summary, we obtained the 58 related genes most closely associated with BC by constructing a gene co-expression network.

**JAM3 was an independent prognostic factor associated with poor prognosis in BC**

At first, we intersected the 172 DEGs with the 58 related genes from the previous step, and the Venn diagram showed 42 overlapping genes (Figure 1D). Then the 42 intersected genes were analyzed by LASSO regression analysis and finally 16 genes (**HSPB6, DIXDC1, CNN1, SPARCL1, DCN, FLNC, FHL1, BIN1, JAM3, RASL12, PDLIM3, FXYD6, PLA2G4C, TGFB3, COL6A2, PTRF**) were identified (Figure 1E and F). On the one hand, it was clear that all 16 genes had considerably lower expression levels in BC tissues than in normal tissues in our GEO cohort, on the other hand, the accuracy of these genes as diagnostic genes was quite high, with all AUC values of more than 0.79, including JAM3 (Figure 1G, H, Supplementary material 1, 2). A similar trend was observed in the TCGA cohort, except for gene **PTRF**. The remaining 15 genes also exhibited lower expression in BC with high diagnostic values accuracy (Supplementary Figure 2F, G, Supplementary material 3, 4).

To further investigate the effect of these 15 genes on the prognosis, we performed univariate and multivariate cox regression analysis on BC samples from the TCGA cohort by using the survival package of R. From the results we found a close correlation between JAM3, age, and stage and the survival of patients (Figure 2A, B). Besides, JAM3 was the only independent prognostic factor among the 15 genes which acted as a risk factor in BC. Substantially, we divided these BC samples into two groups based on the median of JAM3 expression: the high-expression (HE) group and the low-expression (LE) group.

Next, we conducted Kaplan-Meier analysis to assess overall survival (OS) and progression-free survival (PFS). The Kaplan-Meier curve suggested that the HE group had a much lower
survival rate than the LE group (Figure 2C and D). Meanwhile, ROC curves showed that JAM3 was effective in predicting 1-, 3- and 5-year survival rates, which were 0.551, 0.647 and 0.664, respectively (Figure E). Survival analysis of external datasets (GSE13507, GSE48276, GSE37817) also supported our results (Supplementary Figure 5A). Above all, JAM3 was directly related to the poor prognosis of BC. Interestingly, we found that JAM3 level was decreased in tumor between paired samples based on the TCGA cohort in BC (Figure 2F), while we found a higher methylation level of JAM3 in BC as well, which was consistent with those previous studies, indicating the important impact of methylation to influence the JAM3 functions (Figure 2G). Additionally, we used the website of TIMER 2.0 to check the expression of JAM3 in pan-cancer (Figure 2H), and the results showed that JAM3 was higher expression in 5 types of tumors and lower expression in 12 types of tumors, including BC. Therefore, we believed JAM3 was a potentially prognostic gene to investigate.

Worse clinical parameters were found in BC patients with high expression level of JAM3

To better comprehended the clinical significance of JAM3, we produced a heatmap (Figure 3A) to illustrate the correlation between JAM3 expression level and clinical parameters including age, gender, grade, stage, T stage, N stage, and M stage. Detailed clinical information is displayed in the Supplemental Sheet 1 and Supplemental Table 1. We realized that there were substantial differences between the HE and LE groups in the following traits: tumor grade (grouped by high and low), stage (grouped by I-II, III and IV), T stage (grouped T0-2 and T3-4), N stage (grouped N0-1 and N2-3). As the results shown, high level of JAM3 was closely associated with worse clinical parameters (higher tumor grade, higher T and N stages, as well as higher tumor stage) (Figure 3B-G, Supplementary Figure 6). Meanwhile, we validated this in an external dataset, the GEO dataset (GSE13507, GSE48276, GSE37817), and obtained almost consistent results, in addition, suggesting that the
expression of JAM3 was also significantly elevated in MIBC (Supplementary Figure 5B-H).

These findings indicated that clinical parameters can be effectively distinguished by measuring the expression of JAM3.

Finally, we created a prognostic nomogram by combining clinical features and JAM3 to assess the potential value of clinical application of JAM3, which exhibited an effective result to predict prognosis of BC (Figure 3I). Additionally, we constructed the 1-, 3- and 5-year calibration plots to test the performance of the nomogram model, which showed that our nomogram could predict survival with relative accuracy (Figure 3H). In summary, all the preceding implied that JAM3 has significant clinical application potential.

Biofunction analysis indicated JAM3 was related to calcium signaling pathway and PI3K-Akt signaling pathway, as well as the epithelial–mesenchymal transition (EMT) related proteins.

Moreover, we found there were 2565 DEGs between the HE group and LE group when the threshold was set to FDR < 0.05 and |log2 (FC)|≥1, The heatmap displayed the top 50 greatest significant up- and down-regulated genes (Figure 4A).

Substantially, we applied these JAM3 related DEGs to GO function and KEGG pathway enrichment analysis (qvalue<0.05). In KEGG, we noticed that these DEGs were mainly enriched in the calcium signaling pathway, neuroactive ligand-receptor interaction, ECM-receptor interaction, PI3K-Akt signaling pathway and so on (Figure 4B). In GO enrichment analysis, we noticed the most significant functions were mainly related to extracellular matrix (Figure 4C). Meanwhile, the results of the GSEA analysis showed that calcium signaling pathway, ECM-receptor interaction and neuroactive ligand-receptor interaction were active in the HE group (Figure 4D).
Additionally, according to the comparison of HE group to LE group, we noticed that JAM3 level could influence the expression of several EMT related genes. Among these genes, we found that genes ZEB1, CDH2, MMP2, VIM and SNAI1 were upregulated > 1 logFC with p < 0.05 in HE group. Accordingly, we speculated high level of JAM3 could regulate EMT related proteins, which might be a potential mechanism for prognostic function of JAM3.

**Overexpressed JAM3 promoted the BC cells migration and invasion abilities by regulating EMT process**

Thus, we processed a serial experiment to validate our speculation. Firstly, we examined the fundamental expression level of JAM3 in human normal bladder cell and BC cells. As the results shown, we found the fundamental expression level of JAM3 in BC cell lines T24, UMUC3 and RT112 were all lower than that in SVHUC cell, which was consistent with our result in bioinformatic analysis (Figure 5A, B). Next, we established the overexpressed T24 and UMUC3 cell lines by using overexpressing plasmid of JAM3. After we confirmed the fluorescence percentage was more than 80%, we processed the transwell assay to assess the migration and invasion abilities of overexpressed BC cells. We found that both of overexpressed T24 and UMUC3 cells had higher wound healing rates (Figure 5C-F) in 48 hours experiments, as well as remarkable increase of cell migration and invasion abilities in 24 hours observation (Figure 5G, H). Further, to evaluate the effect of overexpressed JAM3 on EMT related proteins, we extracted cell proteins after 48 hours transfection and processed western blot assay. We found remarkably upregulated level of JAM3 in OE group than NC group in both T24 and UMUC3 cells, as well as higher levels of N-Cadherin and MMP-2 proteins, while expression level of Claudin-1 was apparently inhibited (Figure 5I-L). These findings indicated overexpressed BC cells gained more characters of mesenchymal cells to promote the EMT level, and preliminarily validated our speculation.
JAM3 was related to poor immune functions in BC

To learn more about the connection between JAM3 and tumor immunity, we used the algorithm of cibersort to calculate the infiltration of 22 different immune cell types in each BC sample. Firstly, we performed the difference analysis of 22 types of immune cells based on JAM3 expression levels. The infiltration degree of naive B cells, resting memory CD4 T cells, M2 macrophages and resting Mast cells was higher in the HE group. On the contrary, the infiltration degree of CD8+ T cells, follicular helper T cells and activated Dendritic cells was higher in the LE group (Figure 6A). Even more interestingly, the 4 types of immune cells with higher infiltration in the HE group had a significant positive correlation with JAM3 expression. Likewise, memory B cells and the 3 types of immune cells with higher infiltration in the LE group had a significantly negative correlation with JAM3 expression (Figure 6B), and the scatter plots showed more details of the correlation (Figure 6C-I). These results revealed that JAM3 was related to poor immune cell infiltrations in BC, thus providing some evidence for the relevance of JAM3 to poor patient prognosis.

Additionally, we found a negative correlation (R= -0.12) between tumor mutation burden and JAM3 expression (Figure 6J). Besides, JAM3 was found positively correlated with immune checkpoints CD200, NRP1, TNFSF4, and CD28, with significant values more than 0.4 (Figure 6K). These results suggested that JAM3 could partially predict the effect of immunotherapy to some extent.

Co-expression network of JAM3 in BC

Further, we used the website of string to build a Protein-Protein Interaction Network (PPI) that gives us directions to explore JAM3’s capabilities (supplementary figure3A). A total of 275 genes are strongly correlated with JAM3 (|r| > 0.6) (Supplementary material 5). Next the diagram (supplementary figure3B) showed the 11 genes most strongly correlated with JAM3,
which are negatively correlated with JAM3 were S100A11, NT5C, SYTL1, STXBP2, AC068831.5 and positively correlated with JAM3 were DCHS1, ADGRA2, ZEB1, MAP1A, PRKG1 and ZNF521 which details were showed in the correlation diagrams (supplementary figure3C). Among the genes, MAP1A, ZEB1, and ZNF521 were reported to be associated with poor behavior or poor prognosis in BC[23-25]. These findings will give us clues when we look deeper into JAM3.

**DISCUSSION**

BC is the most frequent malignancy in the urinary system which has been shown to be a heterogeneous disease, alterations in DNA and RNA may underlie its clinical and pathological features[32,33]. In this study, we identified JAM3 as an independent prognostic factor in BC, but the roles of JAM3 are controversial in multiple cancers with potential prognostic value. In renal cell carcinoma and small cell lung carcinoma, JAM3 played roles as an oncogene to promote tumorigenesis, while the opposite function was observed in colorectal cancer and esophageal cancer[24,25,34,35]. In BC, a multiple gene model was constructed to predict prognosis of BC patients, while JAM3 was a segment of the model which function as a risk factor but without further investigation[36]. According to our results, we proved that high level of JAM3 was a valuable prognostic factor for predicting poor prognosis, worse clinical features and poor immune cell infiltration in BC.

Further, we found that BC cells with overexpressed JAM3 had apparently promoted migration and invasion abilities by processing transwell assay. Meanwhile, we proved that overexpressed JAM3 in BC cell could upregulate N-cadherin and MMP2 proteins, while downregulate Claudin-1 protein as well. The increasing level of N-cadherin was considered as a landmark of epithelial features loss and gain the mesenchymal features, which usually accompanied by decreased E-cadherin level and promoted the tumor invasion abilities[37]. Besides, the cadherin proteins were closely related to calcium ions, which was consistent
with our bioinformatic findings. The higher level of MMP2 could help tumor cell breakthrough the extracellular matrix and promoted tumor invasion abilities[27]. While downregulated level of Claudin-1 could influence the cell-cell adhesion function as well during the EMT process[38]. Similarly, JAM3 was found highly expressed in renal carcinoma cells, inhibited tumor cell apoptosis and promoted cell migration by upregulating levels of N-cadherin, integrin β1 and MMP-2[34]. These findings distinguished that high level of JAM3 promoted the cell migration and invasion by regulating the EMT process in BC, eventually leading to tumor progression and metastasis. Of course, more verification is required because WB is a non-quantitative outcome.

Importantly, the results of GO, KEGG and GSEA analysis showed that these JAM3-related DEGs were mainly concentrated in functions and pathways associated with the calcium signaling pathway, extracellular matrix and PI3K-Akt signaling pathway. It has been demonstrated that calcium ion channels and pumps are abnormally expressed in various tumors, with complex influence on tumor cell proliferation, metastasis, invasion and drug resistance[39]. Also, it is reported that high serum calcium level was proved to be an independent risk factor for bone metastases in BC patients, highlighted the important functions of calcium ion in BC[36]. Extracellular matrix of tumor plays a fundamental and dynamic role in the development of the tumor microenvironment, the growth of tumor cells are often supported by widespread biochemical and biomechanical alterations in the tumor matrix[40,41]. Besides, extracellular matrix also tends to support the tumor's proliferation, migration and suppress anti-tumor immune function in BC[42,43]. In tumor cells, the PI3K-AKT pathway is often over-activated, and the aberrant activation of this pathway is closely related to the occurrence and development of a variety of tumors [44–46], thus becoming an important target in cancer research. The PI3K-AKT pathway plays a key regulatory role in tumor cells, and participates in a number of biological processes such as cell survival,
proliferation, apoptosis and metabolism. And the results of enrichment analysis also provide clues that the role of JAM3 in BC cells, such as promoting EMT, may be accomplished through the PI3K-AKT pathway. This also refers to the next study direction: in BC, JAM3 promotes tumor growth via the PI3K-AKT pathway, including proliferation, migration, and invasion. However, since these analyses are based on public databases, which have certain shortcomings, such as the variability among individual patients, the results may be biased. The precise process by which JAM3 accelerates BC development has to be investigated and confirmed by our tests in the near future. Therefore, it is reasonable to speculate that the risk role of JAM3 in BC is closely related to calcium signaling, the extracellular matrix and PI3K-Akt signaling pathway, which could be the directions for further investigation. If possible, we would like to further validate our conjecture with clinical samples in future studies.

Tumor microenvironment (TME) plays an important role in tumor initiation, progression, invasion and spread[47]. Several articles have provided constructive insights into the development and treatment of BC based on the immune genes and the TME[48–50], and the existence of a close association between JAM3 and a variety of immune cells suggests to us that there exists a certain correlation between JAM3 and the immune microenvironment of bladder cancer, which we believe can be further elucidated in the future studies.

Consistently, we found that high level of JAM3 was positively associated with infiltration levels of resting Mast cells and M2 Macrophages. M2 macrophages promote tumor angiogenesis and tumor cell development, blocking the function of T cells and are associated with poor prognosis, also in BC[51,52]. While, Mast cells exert pro- or anti-tumor effects due to different tumors including BC and different tumor microenvironments[53,54]. Meanwhile, a significant negative correlation with activated Dendritic cells and CD8+ T cells was observed as well. Activation of CD8+ T cells was crucial in tumor immunity, but a key problem with tumor antigen presentation for effective antitumor response is dendritic cells
must effectively take up and present tumor antigens and subsequently activate CD8 + T cells[55]. In addition, JAM3 was associated with the CD200, an inhibitory immune checkpoint[56] that suppressed anti-tumor immune function by binding its receptor CD200R on myeloid cells[57,58]. These immunological aspects of the analysis above corroborate to some extent the association of JAM3 with the tumor microenvironment in BC.

Undeniably, there are some drawbacks in our work, including ethnic variations in the GEO and TCGA datasets, insufficient sample size, and a lack of deeper mechanistic investigation. Therefore, the roles of JAM3 in BC needs to be further investigated.

CONCLUSION

In conclusion, we identified high level of JAM3 is a valuable independent factor for predicting poor prognosis by regulating EMT process, as well as predicting the bad immune infiltrations, which provides a new biomarker for determining the prognosis and immune functions of BC patients.

ACKNOWLEDGMENTS

We appreciate TCGA data portal and GEO data portal for providing data of bladder cancer.

REFERENCES


Figure 1. Construction of BC-related gene co-expression modules and gene screening. (A) Heatmap of the correlation between module eigengenes and clinical traits of BC. (B) Distribution of average gene significance and errors in the modules associated with BC. (C) Scatter plot of module eigengenes related to BC in the turquoise module. (D) Venn plot showed 42 overlapping genes between 172 DEGs and 58 genes which filter from WGCNA. (E) Tenfold cross-validation for the 42 overlapping genes in the LASSO analysis. (F) LASSO
coefficient profiles of 42 overlapping genes for BC. (G) Boxplot of JAM3 expression across BC and normal samples in GEO cohort. (H) ROC curve of JAM3 as a diagnostic gene for BC in GEO cohort.

Figure 2. Association of JAM3 with prognosis of BC. (A, B) Univariate and multivariate Cox regression analysis. (C, D) Kaplan-Meier analysis for OS and PFS of JAM3 in BC. (E) Time-dependent ROC curves and area under the curves (AUCs) at 1-, 3-, 5-year were used to evaluate the predictive value of JAM3. (F) Paired expression analyses of JAM3 in TCGA cohort for BC. (G) Difference in M6A methylation level of JAM3 between BC and normal samples. (H) The expression of JAM3 in pan-cancer.
Figure 3. Relationship between JAM3 expression level and clinical parameters. (A)
Difference analysis of clinical traits in high and low JAM3 expression groups. (B-G)
Difference analysis of the JAM3 expression in different clinical traits. (I) Nomogram was
established to predict the risk score and survival probability of BC patients. (H) 1-, 3-, 5-year calibration diagrams describe nomogram performance.

Figure 4. Enrichment analysis about the differential genes between HE and LE groups. (A) Heatmap showed the 100 genes with the most significant differences. (B, C) GO analysis and KEGG analysis revealed the potential biological functions and pathways involve in JAM3. (D) GSEA analysis showed the active pathways in HE and LE groups.
Figure 5. Overexpressed JAM3 promoted BC cell migration and invasion abilities by regulating expression levels of EMT related genes. (A, B) The fundamental expression of JAM3 in normal bladder cells and BC cells. (C, D) Wound healing assay for overexpressed T24 cells (C) and UMUC3 cells (D). (E, F) Statistical results of wound healing assay for T24 cells (E) and UMUC3 cells (F). (G, H) Migration and invasion assay for overexpressed T24 cells (G) and UMUC3 cells (H). (I, J) Overexpressed JAM3 upregulated levels of N-Cadherin and MMP-2 proteins, while inhibited the level of Claudin-1 protein in T24 cells (I) and UMUC3 cells (J). (K, L) The relative protein expression level of EMT related proteins, calculation followed protocol as below: gray levels of targeted proteins divided by gray levels of paired tubulin protein. NC: Negative Control group. OE: Overexpressed group.
Figure 6. Correlation analysis between JAM3 and tumor-infiltrating immune cells. (A) The difference of 22 immune cell infiltration between HE and LE groups. (B) Relationships between the expression of JAM3 and 22 types of tumor-infiltrating immune cells. (C-I) Correlation of JAM3 expression with naive B cells, resting memory CD4 T cells, M2 macrophages, resting Mast cells CD8 T cells, follicular helper T cells and activated Dendritic cells. (J) Relationship between JAM3 expression and tumor mutation burden. (K) Correlation between JAM3 and immune checkpoints.
Figure S1. DEGs and their enrichment analysis. (A, B) The volcano plot for 172 DEGs and the heat map for most relevant 100 DEGs (24 up-regulated and 148 down-regulated). (C, D) Bar plot of GO and KEGG analyses for DEGs. (E) Bubble plot of DO analysis for DEGs. (F, G) GSEA analysis in BC and normal samples.
Figure S2. Construction of BC-related gene co-expression modules. (A) Clustering dendrogram and trait indicator of 481 samples. (B) Analysis of the scale-free fit index and the mean connectivity for various soft-thresholding powers. (C) Scale free topology when the power value was seven. (D) Hierarchical clustering analysis of WGCNA modules. (E) Dendrogram of all genes clustered based on a dissimilarity measure (1 - TOM). (F) Boxplot of JAM3 expression across BC and normal samples in TCGA cohort. (G) ROC curve of JAM3 as a diagnostic gene for BC in the TCGA cohort.
**Figure S3.** Co-expression network of JAM3 in BC. (A) Protein-protein interaction network for JAM3. (B) The top five genes negatively correlated with JAM3 and the top six genes positively correlated with JAM3. (C) The top six strongest positively co-expressed genes.
### Figure S4

Demonstration of clinical traits in the GEO datasets (GSE3167, GSE13507, GSE52519, GSE65635, GSE100926, GSE120736).
Figure S5. (A) Kaplan-Meier analysis of JAM3 in BC based on GSE13507, GSE48276, GSE37817. (B-H) Relationship between JAM3 expression level and clinical parameters based on GSE13507, GSE48276, GSE37817.
Supplementary Figure 6. (A-C) Relationship between JAM3 expression level and clinical parameters based on TCGA.

Supplementary material 1. Boxplot of 15 genes' expression across BC and normal samples in GEO cohort.

Supplementary material 2. ROC curves of 15 genes as the diagnostic genes for BC in GEO cohort.

Supplementary material 3. Boxplot of 14 genes' expression across BC and normal samples in TCGA cohort.

Supplementary material 4. ROC curves of 14 genes as the diagnostic genes for BC in the TCGA cohort. Supplementary material 5. 275 genes which strongly correlated with JAM3.

Supplementary Sheet 1: Clinical data including survival data on patients with bladder cancer from TCGA.

Supplementary Table 1: Clinical data on patients with bladder cancer from TCGA.