High expression of *EMP1* predicts a poor prognosis and correlates with immune infiltrates in bladder urothelial carcinoma

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Abstract. Epithelial membrane protein 1 (*EMP1*) is a key gene that regulates cell proliferation and metastatic capability in various types of cancer, and serves an important role in tumor-immune interactions. However, the association between *EMP1* and clinical prognosis, as well as the presence of tumor-infiltrating lymphocytes in bladder urothelial carcinoma (BLCA) remains unclear. The present study aimed to explore the relationship between *EMP1* expression and tumor immune cell infiltration in BLCA. In the present study, *EMP1* expression in BLCA was analyzed using the Oncomine database, The Cancer Genome Atlas (TCGA) and the Tumor Immune Estimation Resource (TIMER). The effects of *EMP1* on clinical prognosis were evaluated using the Kaplan-Meier plotter and Gene Expression Profiling Interactive Analysis. The correlations between *EMP1*, cancer immune infiltrates and lymphocyte abundance were determined using the TIMER and Tumor immune system interaction database. In addition, correlations between *EMP1* expression and gene markers in immune infiltrates were analyzed using cBioportal. The results demonstrated that, compared with adjacent normal tissues, *EMP1* was downregulated in BLCA tissues. High expression of *EMP1* was significantly associated with poor overall survival (OS) in BLCA cases obtained from TCGA. Multivariate Cox analysis revealed that *EMP1* was an independent predictor of OS in patients with BLCA. Gene set enrichment analysis revealed that *EMP1* was associated with cancer-related pathways and was positively correlated with the levels of infiltrating $CD8^+$ T cells, macrophages, neutrophils and dendritic cells in BLCA. Further analysis demonstrated that *EMP1* was significantly associated with the enrichment of multiple types of lymphocyte. *EMP1* expression exhibited a strong correlation with a range of immune markers in BLCA. In conclusion, the results of the present study demonstrated that *EMP1* was associated with a poor prognosis in patients with BLCA, and that the levels of immune infiltration and multiple immunomarker groups were associated with *EMP1* expression. These results suggested that *EMP1* may be used as a predictive biomarker to determine the prognosis and immune infiltration in BLCA.

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Introduction

The incidence of bladder urothelial carcinoma (BLCA) ranks ninth among all malignancies in the global population and fourth among all malignancies occurring in men (1). The main risk factors of BLCA are cigarette smoking, exposure to toxic industrial chemicals and gases, and genetic susceptibility (2). Although standard treatment and supportive care have improved the overall survival (OS) and quality of life, the prognosis for patients with BLCA remains poor (3).

Immune-related mechanisms serve an important role in BLCA, and immunotherapeutic strategies are considered to be a promising direction for the treatment of BLCA (4,5). Immunotherapy seeks to manipulate the patient's own immune response to improve the clinical outcome by promoting immune cells that can kill target cancer cells (5). The receptor-ligand pairing of programmed cell death protein-1 (PD-1) has been identified to be a crucial immune checkpoint; however, current immunotherapies using anti-PD-1 have only achieved partial response in patients with advanced BLCA (6,7). In addition, an increasing number of studies have demonstrated that patients with bladder cancer with a high level of tumor-infiltrating lymphocytes exhibit improved survival (8-10). However, studies on the prognostic value of immune cell subsets in patients with BLCA have yields completely opposite results (11). Therefore, there is an urgent need to elucidate the specific immune phenotypes of tumor-immune interactions and identify novel immune-associated therapeutic targets in BLCA.

Epithelial membrane protein 1 (*EMP1*) is a protein-coding gene; its expression and significance in human cancer and its biological effects have been explored *in vitro*, demonstrating that *EMP1* significantly reduces cell migration and invasion, and increases apoptosis and caspase-9 expression in carcinoma of the nasopharynx, stomach, breast and prostate (12-16). By contrast, studies of acute lymphoblastic leukemia (ALL) have revealed that *EMP1* is an indicator of poor prognosis (17).

Limited information is available about the mechanism underlying the effects of *EMP1*. Previous studies have

suggested that *EMP1* works primarily by regulating signal transduction between cells and the extracellular matrix (18). *EMP1* may be associated with the proto-oncogene *c-myc* (19), and other studies have revealed that *EMP1* is regulated by the epidermal growth factor receptor (EGFR) (20,21). In addition, *EMP1* is involved in the tight connection between cells, which may cause the occurrence and development of non-small cell lung cancer by activating the PI3K/AKT pathway (22). Wang *et al* (23), demonstrated that EMP family members and integrins synergistically regulate cell adhesion and migration *in vitro*, and integrin-based cell adhesion leads to autoimmune diseases. Therefore, previous studies have suggested that *EMP1* serves an important role in tumorigenesis and tumor immunity, but the effects of this gene on the OS of patients with BLCA and the underlying function of *EMP1* in tumor-immune interactions remain unclear.

The present study aimed to comprehensively analyze *EMP1* expression and its association with the prognosis of patients with BLCA. In addition, the correlation between *EMP1* and tumor-infiltrating immune cells in the BLCA microenvironment was determined. The correlation between *EMP1* and the immune cell-specific genes reported in literature, as well as immunological checkpoint-specific genes were further studied, and the expression level of *EMP1* in tumor-tissue specimens and adjacent normal tissues of patients with BLCA were compared.

Materials and methods

Patients and tissue samples. Bladder cancer and adjacent normal tissues were collected from patients with BLCA at the Peking University Shenzhen Hospital (Shenzhen, China) between September 2018 and December 2019. The specimens were all collected during bladder cancer resection, and the distance between tumor tissue and adjacent normal tissue was >2 cm. BLCA was diagnosed and classified through pathological examination based on the World Health Organization classification system (24). Specimens from patients with a history of preoperative chemotherapy were excluded. The study protocol was approved by the Ethics Committees for Human Experiments of Peking University Shenzhen Hospital. All patients signed an informed consent form before sample collection.

Image processing. The paraffin-embedded tumor sections (5 μm thick) were stained with H&E or antibodies against *EMP1* (cat. no. ab230445; 1:75; Abcam) according to the routine immunohistochemical staining method (25). All images shown are wide-field light microscopy images that were acquired at sufficient resolution.

Acquisition of mRNA data. The gene expression data and corresponding clinical information were downloaded from TCGA website (https://portal.gdc.cancer.gov) for BLCA, and estimated as $\log_2(x+1)$ transformed RSEM normalized counts (26). BLAC samples comprised samples of 404 patients with BCLA, including 28 cases with adjacent non-tumorous tissue as control group. All data were processed using R-studio software (v3.5.3) (27). The 'ESTIMATE' R package was used to predict the presence of infiltrating stromal/immune cells in tumor tissues using gene expression data (28).

Oncomine database analysis. The levels of *EMP1* gene expression in various types of cancer were identified using the Oncomine database (https://www. oncomine.org). The threshold was determined according to the following values: P-value of 0.001 and fold-change of 2.

Kaplan-Meier plotter database analysis. The Kaplan Meier plotter (http://kmplot.com/analysis/) is capable of assessing the effect of 54,000 genes on patient survival in 21 types of cancer (29). The association between *EMP1* expression and survival in patients with BLCA was analyzed using the Kaplan-Meier plotter. The hazard ratio (HR) with 95% confidence intervals (CIs) and log-rank P-value were computed.

Tumor Immune Estimation Resource (TIMER) database analysis. TIMER (https://cistrome. shinyapps.io/timer/), which is a comprehensive resource for the systematic analysis of immune infiltrates across various types of cancer, was used in the present study to analyze the level of *EMP1* expression in BLCA and the correlation between *EMP1* expression and the abundance of immune infiltrates, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages and dendritic cells via gene modules. The immune cell infiltration score of each patient in TCGA database was obtained using TIMER, and the patients were divided into high and low score groups based on the median value.

Immunological analysis by Tumor immune system interaction database (TISIDB). The correlations between the abundance of tumor-infiltrating lymphocytes and *EMP1* expression were analyzed using TISIDB (http://cis.hku.hk/TISIDB), which is a web portal for tumor and immune system interactions integrating multiple heterogeneous data types (30). In the present study, the enrichment data of 28 immune cells provided by TISIDB, including activated $CD8^+$ cells (Act CD8), central memory CD8 cells (Tcm CD8), effector memory CD8 cells (Tem CD8), activated $CD4^+$ cells (Act CD4), central memory CD4 cells (Tcm CD4), effector memory CD4 cells (Tem CD4), T follicular helper cells (Tfh), gamma delta T cells (Tgd), type 1 T helper cells (Th1), type 17 T helper cells (Th17), type 2 T helper cells (Th2), regulatory T cells (Treg), activated B cells (Act B), immature B cells (Imm B), memory B cells (Mem B), natural killer (NK) cells, CD56 bright NK cells (CD56bright), CD56 dim NK cells (CD56dim), myeloid derived suppressor cells (MDSCs), NK T cells (NKT), activated dendritic cell (Act DCs), plasmacytoid DCs (pDCs), immature DCs (iDCs), macrophages, eosinophils, mast cells (Mast), monocytes and neutrophils, were used to calculate the relationship with the expression of *EMP1* in BLCA.

Gene expression and survival analysis in Gene Expression Profiling Interactive Analysis (GEPIA). The online database GEPIA (http://gepia.cancer-pku.cn/index.html) was used to analyze the differential expression of *EMP1* and its prognostic values.

Co-expression analysis in cBioPortal. The cBioPortal for Cancer Genomics (https://www.cbioportal.org) is an open-access, open-source resource for interactive exploration of multidimensional cancer genomics datasets (31,32) that was used in the present study to determine the correlations

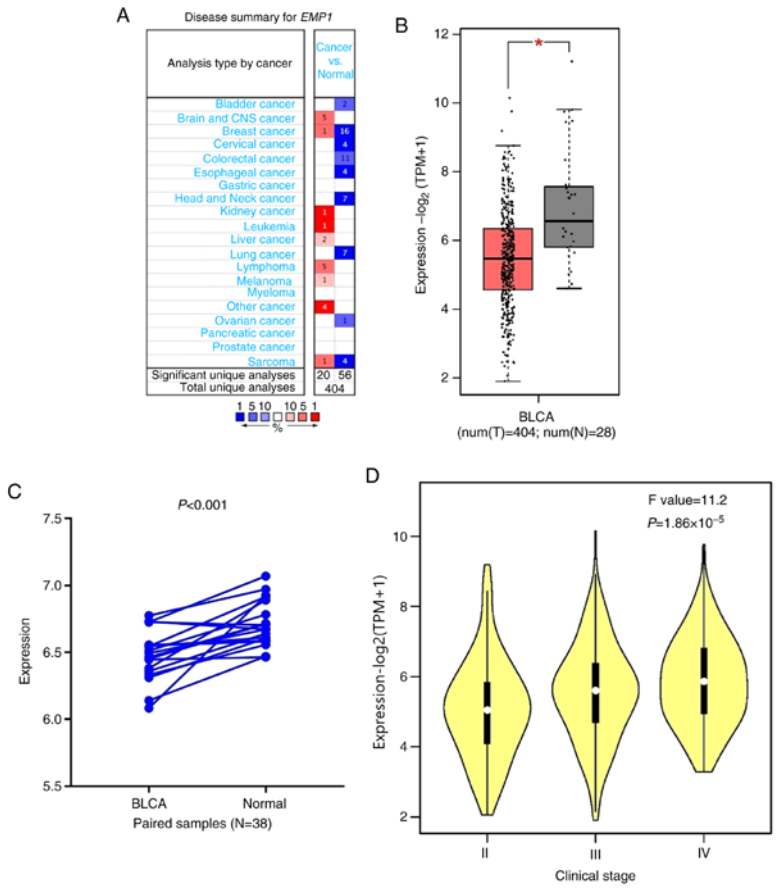

Figure 1. EMP1 expression levels in BLCA and other types of cancer. (A) EMP1 expression in datasets of various types of cancer in the Oncomine database. Cell color is determined by the best gene rank percentile for the analyses within the cell. Blue indicates upregulated EMP1 expression, and red indicates that the gene is downregulated. (B) EMP1 expression was compared between BLCA tissues and normal tissues from The Cancer Genome Atlas using Gene Expression Profiling Interactive Analysis. EMP1 expression in BLCA (n=404) was significantly lower compared with that in adjacent non-tumor tissues (n=28). (C) The level of EMP1 expression was compared between paired samples of BLCA and adjacent normal tissues of the same patient; EMP1 expression was significantly downregulated in BLCA compared with that in adjacent normal tissues. (D) The expression of EMP1 increases with clinical stage. The white dot in the center of the violin plot represents the median of EMP1 expression, and the black box represents the interquartile range. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1.

between *EMP1* expression and tumor-infiltrating immune cell markers. The gene markers of the tumor-infiltrating immune cells included markers of $CD8^+$ T cells, T cells (general), B cells, monocytes, tumor associated macrophages (TAMs), M1 macrophages, M2 macrophages, neutrophils, NK cells, DCs, Th1 cells, Th2 cells, follicular helper T (Tfh) cells, Th17 cells, Tregs and exhausted T cells. The Spearman rank correlation analysis was used to determine the correlation coefficient. *EMP1* expression was plotted on the x-axis, and the expression levels of other genes of interest were represented on the y-axis.

Gene set enrichment analysis (GSEA). To identify the potential mechanisms underlying the effects of *EMP1* expression on BLCA prognosis, GSEA was performed to detect whether an a priori defined set of genes exhibited statistically significant differential expression between the high and low *EMP1* expression groups. Gene sets with a P-value <0.05 and false discovery rate (FDR) <0.25 in the enrichment of MSigDB Collection (c2.cp.kegg.v6.2. symbols) were considered to be significantly enriched.

Statistical analysis. Survival curves were generated using the GEPIA and Kaplan-Meier databases. T test and paired t test were implemented in prism and R software, and the results were displayed in the form of pictures and tables after sorting out the results. Receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to demonstrate the predictive ability of *EMP1* for 3- and 5-year OS. The results generated in Oncomine are presented as P-values, fold-changes and ranks. The results of the Kaplan-Meier plots and GEPIA are displayed with HR and P or Cox P-values from a log-rank test. Univariate Cox analysis was performed to select the potential prognostic factors, and multivariate Cox analysis was performed to verify the association between *EMP1* expression and survival along with other clinical features. P<0.05 was considered to indicate a statistically significant difference.

Results

Association between EMP1 expression and clinicopathologic variables in BLCA. As presented in Fig. 1A, *EMP1* expression was upregulated in brain, breast, kidney and liver cancer, as well as in leukemia, lymphoma, melanoma and sarcoma compared with that in normal tissues. In addition, downregulation of *EMP1* was observed in bladder, breast, cervical, colorectal, esophageal, head and neck, lung, ovarian

Table I. Logistic regression of the expression of EMP1 and the clinicopathological characteristics of patients with bladder urothelial carcinoma.

Characteristic	Total	Odds ratio in EMP1 expression	P-value
Age, years (≥60 vs. <60)	407	1.64 (1.02-2.67)	0.04[a]
Subtype (papillary vs. non-papillary)	402	0.65 (0.42-0.98)	0.04a
Sex (male vs. female)	407	0.80 (0.51-1.23)	0.30
Lymphovascular invasion (positive vs. negative)	273	1.29 (0.80-2.09)	0.30
Recurrence (yes vs. no)	371	1.23 (0.79-1.89)	0.36
Pathologic T classification (III-IV vs. I-II)	374	1.80 (1.17-2.81)	$8.4x10^{-3a}$
Pathologic M classification (M+ vs. M0)	200	2.43 (0.66-11.56)	0.21
Pathologic N classification (N0 vs. N+)	367	0.90 (0.59-1.38)	0.64
Pathologic stage (III-IV vs. I-II)	405	1.496 (0.984-2.283)	0.06
Stromal score (high vs. low)[b]	408	1.65 (0.25-0.56)	$1.91x10^{-6a}$
Immune score (high vs. low)[b]	408	1.54 (0.29-0.64)	$2.89x10^{-5a}$

[a]P<0.05. [b]Grouped according to the median value. EMP1, epithelial membrane protein 1.

cancer and sarcoma in a number of data sets. Differential expression was observed between tumor and normal tissues for *EMP1* in BLCA data from TCGA. The results indicated that *EMP1* was upregulated in BLCA compared with adjacent normal tissues (P<0.001; Fig. 1B) and with paired adjacent healthy tissues (P<0.001; Fig. 1C). In addition, the expression of *EMP1* significantly increased with clinical stage (P<0.001; Fig. 1D). The expression data of *EMP1* are presented in the supplementary materials (Table SI and Table SII).

Upregulation of *EMP1* was significantly associated with advanced age (≥60 vs. <60, P=0.040), histological subtype (papillary vs. non-papillary, P=0.043), pathologic T classification (III-IV vs. I-II, P=0.008), immune score (high vs. low, P<0.001) and stromal score (high vs. low, P<0.001; Table I). However, no significant associations between *EMP1* expression and sex, lymphovascular invasion, tumor recurrence, pathologic M/N classification and pathologic stage were observed.

Survival outcomes and multivariate analysis. The analysis of BLCA cases in TCGA revealed that the 5-year OS of the high *EMP1* expression group was significantly lower compared with that of the low expression group (P<0.001; Fig. 2A). Receiver operating characteristic (ROC) curve analysis demonstrated the predictive ability of *EMP1* for 3- and 5-year OS with the area under the curve (AUC) of 0.737 and 0.739, respectively (Fig. 2B). The association between *EMP1* expression and survival outcome was further confirmed by Kaplan-Meier survival analysis (Fig. 2C). In addition, Fig. 2D-F demonstrated the relationship between *EMP1* and OS in stage II-IV patients. The results showed that *EMP1* overexpression was significantly associated with the poor prognosis of patients with stages II and IV.

The univariate analysis revealed that high *EMP1* expression was significantly associated with poor OS time (HR, 8.93; 95% CI, 3.71-21.47; P<0.001). Other clinicopathological characteristics associated with worse survival included age, pathologic stage, $CD8^+$ T cell, macrophage infiltration, and stomal-score. In the multivariate analysis, *EMP1* remained associated with poor OS (HR, 6.61; 95% CI, 2.39-18.30; P<0.001) in conjunction with advanced age, pathologic stage, and macrophage infiltration (Table II). The data used for multivariate Cox regression are presented in the supplementary materials (Table SIII).

GSEA identifies an EMP1-related signaling pathway. The most significantly enriched signaling pathways were selected based on their normalized enrichment score. As demonstrated in Table III, pathways such as 'pathways in cancer', 'adherens junction', 'neurotrophin signaling pathway', 'endometrial cancer and focal adhesion' were differentially enriched in the high *EMP1* expression phenotype. These signaling pathways may be the mechanisms involved in *EMP1* function.

EMP1 expression is associated with the level of immune infiltration in BLCA. The level of *EMP1* expression correlated with high levels of immune infiltration in five types of immune cells and tumor purity in the TIMER dataset. *EMP1* expression was significantly correlated with tumor purity and infiltration of B cells, $CD8^+$ T cells, macrophages, neutrophils and DCs in BLCA as shown in Fig. 3. These results suggested that *EMP1* may serve a specific role in immune infiltration in BLCA. In addition, the survival analysis from TIMER dataset also showed that high levels of infiltrating $CD8^+$ T cells were significantly associated with poor OS in patients with BLCA (P=0.006), and high expression of *EMP1* predicted poor OS (P<0.01; Fig. 4).

Association between the abundance of tumor-infiltrating lymphocytes and EMP1 expression. The results obtained from TISIDB demonstrated that *EMP1* expression was strongly associated with the abundance of Tcm CD8 and neutrophil cells (both correlation coefficients >0.5 and P<0.05), and moderately related with the abundance of Mem B, Act CD4, Tcm CD4, Tem CD8, Act DC, pDC, iDC, NK, NKT, eosinophil, Tfh, Tgd, Th1, Th2, Treg, macrophage, mast and MDSC (all correlation coefficients between 0.3-0.5 and all P<0.05) (Fig. 5).

Correlation between EMP1 expression and immune markers. The correlations between *EMP1* expression and immune

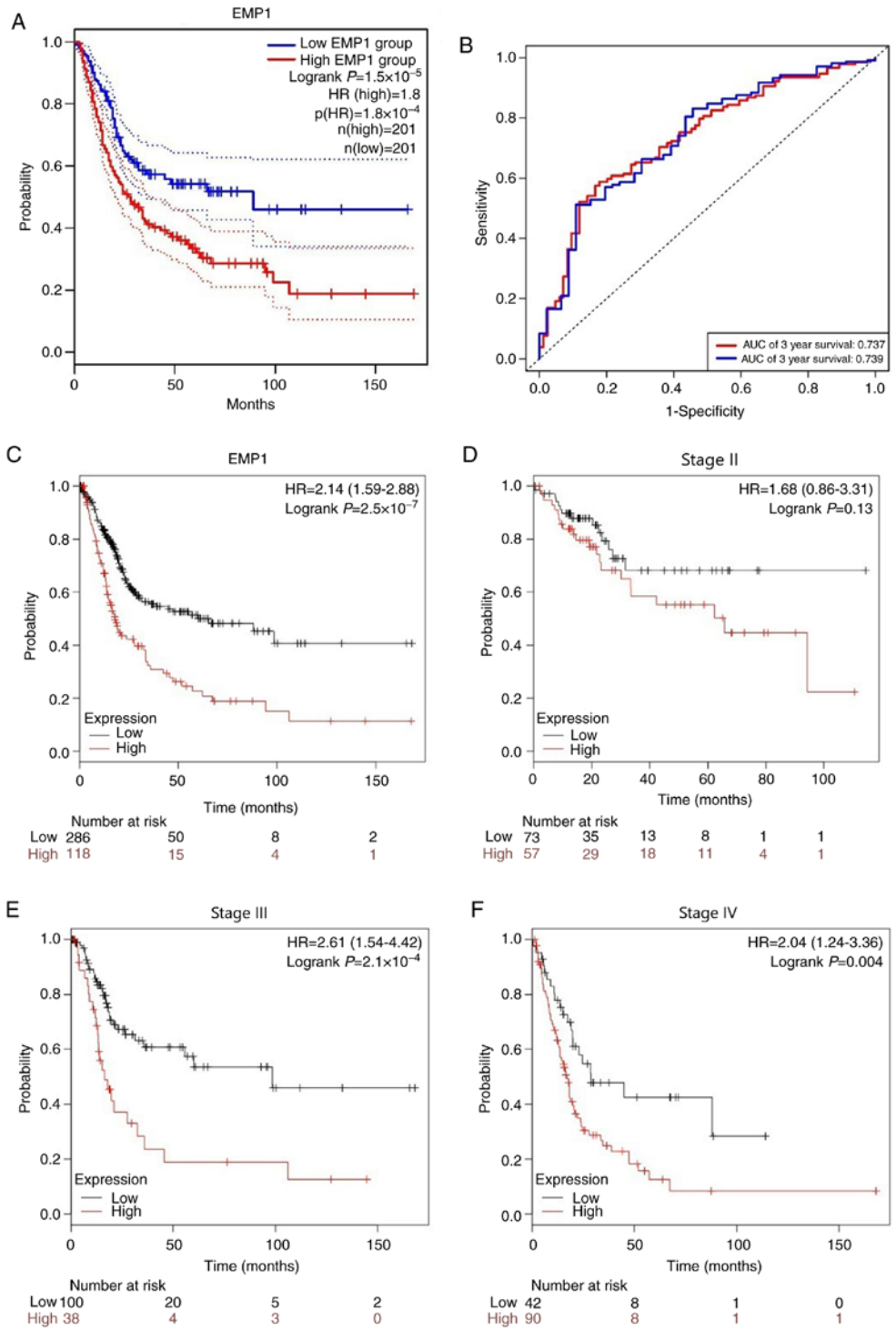

Figure 2. Association between EMP1 expression levels and the OS rate in patients with BLCA. (A) According to the median EMP1 expression level, patients with BLCA were divided into two groups with high and low expression, with 201 patients per group. High EMP1 expression predicted a poor OS rate based on The Cancer Genome Atlas data. (B) Receiver operating characteristic curve demonstrated the predictive ability of EMP1 in BLCA. (C) The OS results calculated by the Kaplan Meier plotter database; patients with low EMP1 expression group exhibited an improved OS rate. (D-F) Kaplan-Meier curves for OS in BLCA cases with clinical stages II-IV. The results demonstrated that the prognosis of the low EMP1 expression group was improved compared with that of the high expression group regardless of clinical stage. Moreover, there was a statistically significant difference in OS between the high and low expression groups in patients with tumors of clinical stages III and IV. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1; OS, overall survival; AUC, area under the curve.

marker genes of the different immune cells, including $CD8^+$ T cells, T cells (general), B cells, monocytes, TAMs, M1 and M2 macrophages, neutrophils, NK cells and DCs are presented in Table IV. The results revealed that the level of *EMP1* expression was significantly correlated with immune markers of various immune cells. The expression levels of the majority of marker sets of monocytes, TAMs and M2 macrophages exhibited a significant correlation with *EMP1* expression. In particular, chemokine (C-C motif) ligand (*CCL*)-2, *CD68*, interleukin 10 (*IL10*) of TAMs, prostaglandin-endoperoxide

Table II. Univariate and multivariate analysis of overall survival using the Cox proportional hazard regression model (N=397).

	Univariate analysis			Multivariate analysis		
Parameter	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.033	1.017-1.049	$4.67x10^{-5}$[a]	1.029	1.012-1.046	$5.0x10^{-4}$[a]
Subtype[c]	0.650	0.454-0.929	0.018[a]	0.814	0.558-1.187	0.286
Sex	0.880	0.633-1.224	0.449	0.862	0.611-1.215	0.395
Pathologic stage	2.240	1.538-3.262	$2.6x10^{-5}$[a]	1.880	1.261-2.802	0.002[a]
B cell	0.0741	0.007-0.754	0.028	0.116	0.010-1.269	0.078
$CD4^+$ T cell	0.286	0.042-1.954	0.202	1.415	0.047-42.416	0.841
$CD8^+$ T cell	5.701	1.430-22.730	0.014[a]	5.243	0.407-67.569	0.204
Neutrophil	0.652	0.076-5.614	0.697	0.006	0.000-0.737	0.037[a]
Macrophage	18.825	4.514-78.518	$5.62x10^{-5}$[a]	8.867	1.506-52.216	0.016[a]
Dendritic	0.898	0.428-1.884	0.776	1.350	0.300-6.039	0.696
Stromal score[b]	0.728	0.530-0.973	0.033[a]	1.043	0.710-1.530	0.832
Immune score[b]	1.072	0.795-1.446	0.649	1.015	0.668-1.542	0.944
EMP1	8.927	3.713-21.466	$1.01x10^{-6}$[a]	6.614	2.390-18.301	$2.75x10^{-4}$[a]

[a]P<0.05. [b]Grouped according to the median value. [c]Papillary vs. non-papillary subtype. EMP1, epithelial membrane protein 1; HR, hazard ratio; CI, confidence interval.

Table III. Gene sets enriched analysis of upregulated EMP1 in BLCA.

NAME	NES	P-value
KEGG ECM RECEPTOR INTERACTION	1.696	0.000
KEGG FOCAL ADHESION	1.795	0.000
KEGG PATHWAYS IN CANCER	1.592	0.002
KEGG NEUROTROPHIN SIGNALING PATHWAY	1.595	0.006
KEGG AXON GUIDANCE	1.612	0.008
KEGG CELL ADHESION MOLECULES CAMS	1.547	0.008
KEGG GAP JUNCTION	1.527	0.010
KEGG ENDOMETRIAL CANCER	1.625	0.017
KEGG HEMATOPOIETIC CELL LINEAGE	1.484	0.019
KEGG NICOTINATE AND NICOTINAMIDE METABOLISM	1.523	0.023
KEGG ADHERENS JUNCTION	1.753	0.024
KEGG GLIOMA	1.559	0.027
KEGG RENAL CELL CARCINOMA	1.530	0.046
KEGG FC GAMMA R MEDIATED PHAGOCYTOSIS	1.536	0.046

Gene sets with P<0.05 and false discovery rate <0.25 were considered significant. NES, normalized enrichment score. EMP1, epithelial membrane protein 1; BLCA, bladder urothelial carcinoma.

synthase 2 (*PTGS2*), interferon regulatory factor 5 (*IRF5*) of the M1 phenotype, *CD163*, V-set and immunoglobulin domain containing 4 (*VSIG4*), and membrane spanning 4-domains A4A (*MS4A4A*) of the M2 phenotype significantly correlated with *EMP1* expression in BLCA (P<0.05), suggesting that *EMP1* may regulate macrophage polarization. High *EMP1* expression is was associated with a high level of DC infiltration in BLCA; DC markers, such as Major Histocompatibility Complex, Class II, DP Beta 1 (*HLA-DPB1)* and Integrin Subunit Alpha X (ITGAX) also exhibited a significant correlation with *EMP1* expression. In addition, for Tregs, a moderate positive correlation was observed between forkhead box P3 (*FOXP3*), C-C Motif Chemokine Receptor 8 (CCR8) and *EMP1* in BLCA. Therefore, these results further confirmed that *EMP1* was associated with infiltrating immune cells in BLCA, which suggested that *EMP1* may serve a crucial role in immune enhancement in the BLCA microenvironment.

EMP1 expression and immune checkpoint correlation analysis. The correlations between *EMP1* and the specific genes of the

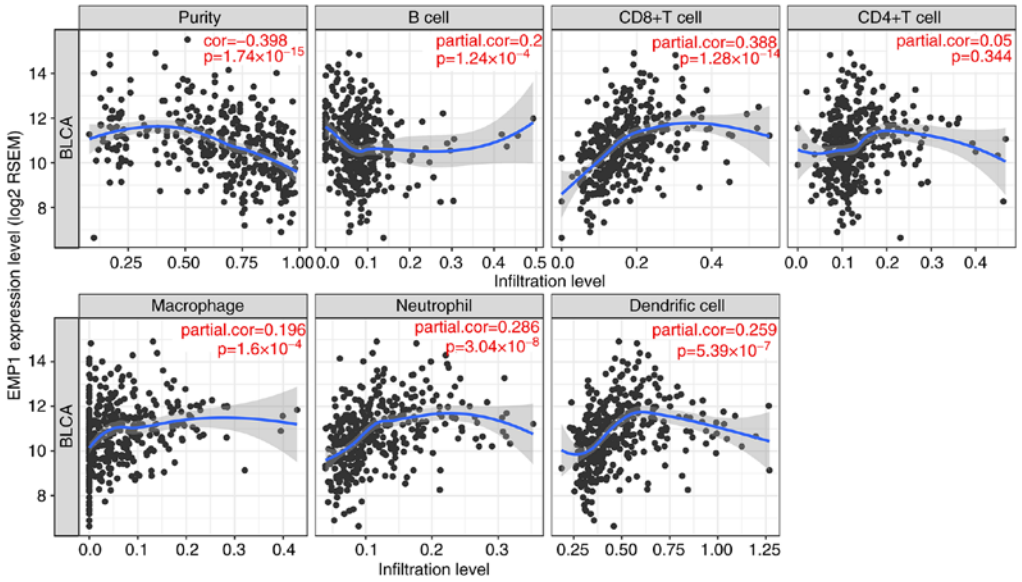

Figure 3. Correlations between EMP1 expression level and BLCA immune infiltration obtained from the Tumor Immune Estimation Resource database.

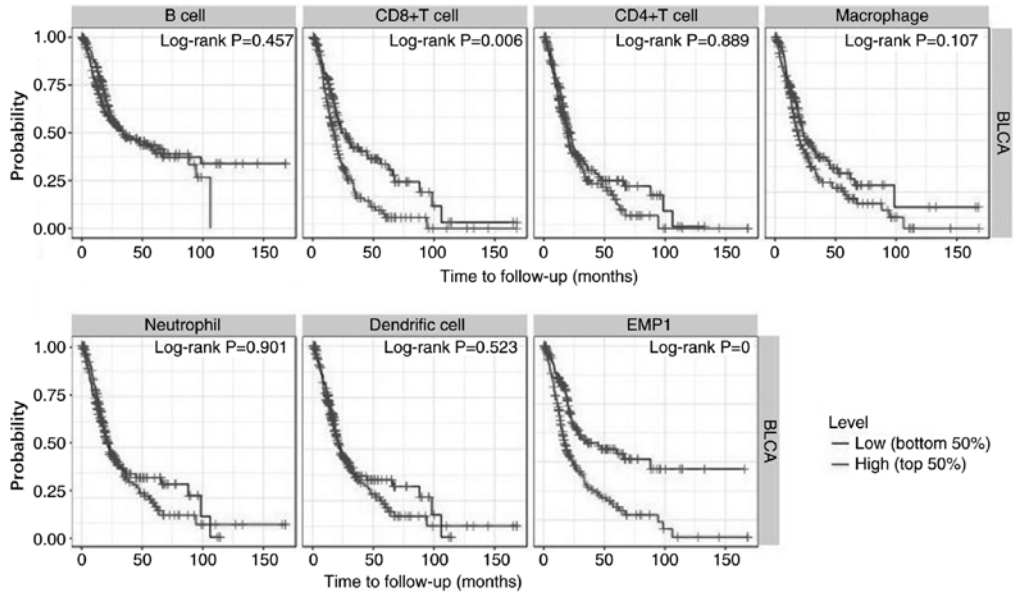

Figure 4. Effects of multiple tumor immune subsets and EMP1 on the prognosis of BLCA patients. The level of $CD8^+$ cell infiltration as well as EMP1 expression were significantly associated with poor OS in all BLCA cases. However, there was no significant association between the infiltration of B cells, $CD4^+$ T cells, macrophages, neutrophils or dendritic cells and OS. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1; OS, overall survival.

immune checkpoints that have been reported in the literature were further assessed. Programmed cell death 1 ligand 1 (*CD274*), programmed cell death 1 ligand 2 (*PDCD1LG2*), hepatitis A virus cellular receptor 2 (*HAVCR2*), cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), lymphocyte-activating 3 (*LAG3*), programmed cell death 1 (*PDCD1*) and T cell immunoreceptor with Ig and ITIM domains (*TIGIT*) were selected for analysis as they have been previously reported to be immunological checkpoint-specific genes. *EMP1* was significantly associated with the expression of these genes ($P<0.05$; Fig. 6).

Expression of EMP1 in BLCA specimens and adjacent normal tissue. Bladder cancer specimens from eight patients with pathologically confirmed BLCA were analyzed in the present study. The patients were all male, and the age range was 66-82 years. Immunohistochemical staining of BLCA tissue specimens and adjacent normal tissues revealed that *EMP1* was strongly stained in adjacent normal tissues, but was the staining intensity was lower in tumor tissues (Fig. 7).

Discussion

The present study examined the levels of *EMP1* expression and the systematic prognostic landscape in BLCA using independent datasets in the Oncomine and TCGA databases. Differential levels of *EMP1* expression between cancer and normal tissues were observed. Consistent prognostic associations of *EMP1* expression in BLCA were identified, in

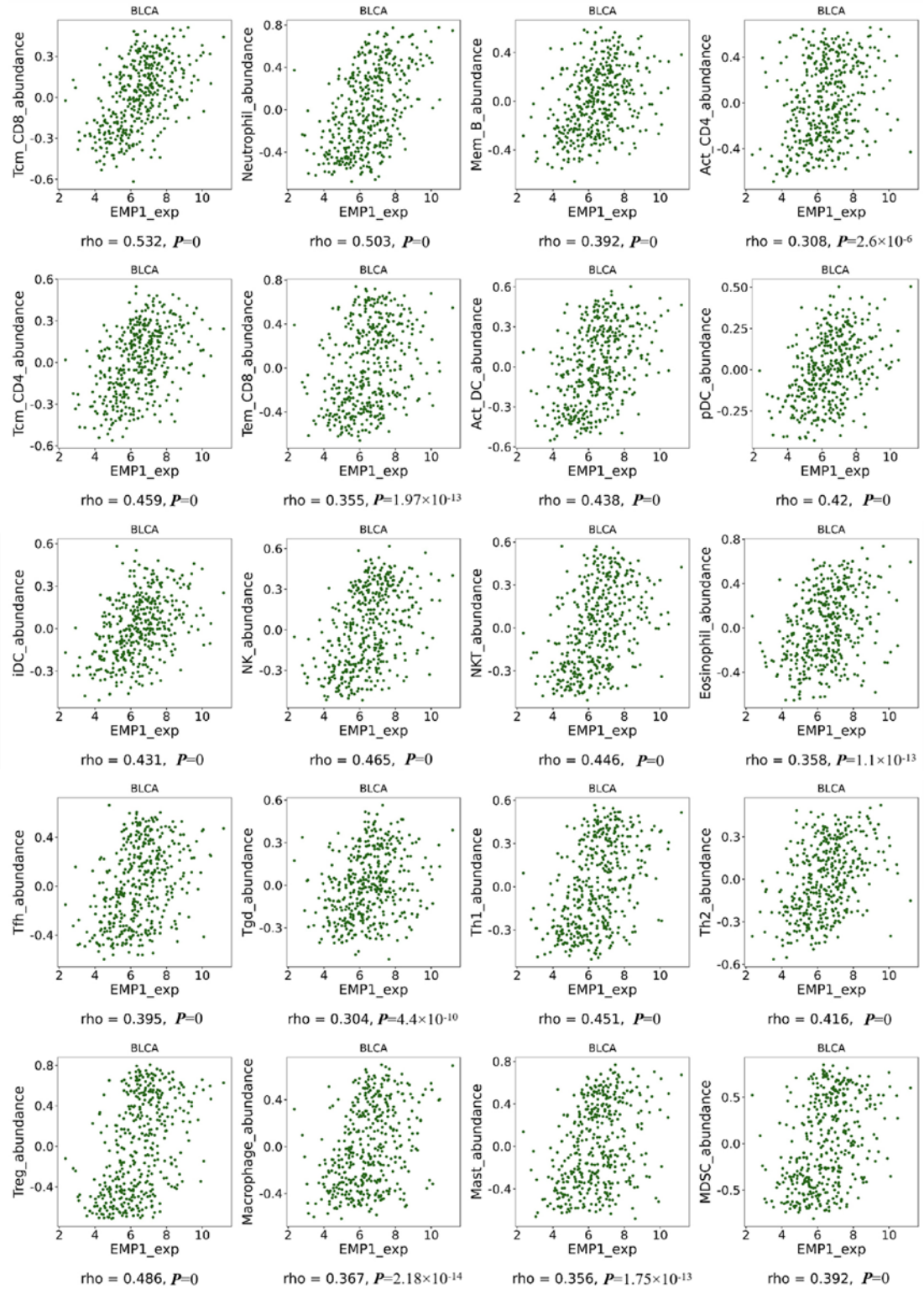

Figure 5. Correlations between EMP1 expression levels and lymphocyte abundance in BLCA using Tumor immune system interaction database. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1; Tcm CD8, central memory CD8 cells; Mem B, memory B cells; Act CD4, activated $CD4^+$ cells; Tcm CD4, central memory CD4 cells; Tem CD8, effector memory CD8 cells; Act DCs, activated dendritic cell; pDCs, plasmacytoid DCs; iDCs, immature DCs; NK, natural killer cells; NKT, NK T cells; Tfh, T follicular helper cells; Tgd, gamma delta; Th1, type 1 T helper cells; Th2, type 2 T helper cells; Treg, regulatory T cells; MDSCs, myeloid derived suppressor cells; Mast, mast cells.

which high levels of *EMP1* expression were associated with a poor OS rate, and further analysis using the Kaplan-Meier plotter revealed that *EMP1* overexpression was associated with poor BLCA prognosis in stages III to IV. The results of the TIMER database analysis demonstrated significant positive correlations between the levels of *EMP1* expression and the infiltration of $CD8^+$ T cells, macrophages, neutrophils and DCs in BLCA; however, *EMP1* expression was

Table IV. Correlation analysis between EMP1 and the genes and markers of immune cells in cBioportal.

Cell type	Gene	Spearman's correlation	P-value
$CD8^+$ T cell	*CD8A*	0.214	$1.30x10^{-5a}$
	CD8B	0.092	0.060
	CD80	0.375	$4.48x10^{-15a}$
T cell (general)	*CD3D*	0.160	0.001^a
	CD3E	0.240	$9.70x10^{-7a}$
	CD2	0.207	$2.42x10^{-5a}$
B cell	*CD19*	0.104	0.036^a
	CD79A	0.177	$3.26x10^{-4a}$
Monocyte	*CD86*	0.434	$3.88x10^{-20a}$
	CD115	0.417	$4.11x10^{-17a}$
TAM	*CCL2*	0.342	$1.24x10^{-12a}$
	CD68	0.366	$2.38x10^{-14a}$
	IL10	0.394	$1.25x10^{-16a}$
M1 Macrophage	*NOS2*	0.131	0.008^a
	IRF5	-0.199	$5.37x10^{-5a}$
	PTGS2	0.296	$1.10x10^{-9a}$
M2 Macrophage	*CD163*	0.442	$6.27x10^{-21a}$
	VSIG4	0.436	$2.22x10^{-20a}$
	MS4A4A	0.403	$2.85x10^{-18a}$
Neutrophil	*ITGAM*	0.381	$1.51x10^{-15a}$
	CCR7	-0.141	0.004^a
Natural killer cell	*KIR2DL1*	0.200	$4.87x10^{-5a}$
	KIR2DL3	0.223	$5.35x10^{-6a}$
	KIR2DL4	0.268	$3.92x10^{-8a}$
	KIR3DL1	0.141	0.004^*
	KIR3DL2	0.170	$5.83x10^{-4a}$
	KIR3DL3	0.067	0.179
	KIR2DS4	0.149	0.003^a
Dendritic cell	*HLA-DPB1*	0.279	$9.81x10^{-9a}$
	HLA-DQB1	0.240	$9.04x10^{-7a}$
	HLA-DRA	0.270	$3.06x10^{-8a}$
	HLA-DPA1	0.277	$1.35x10^{-8a}$
	CD1C	0.262	$8.34x10^{-8a}$
	ITGAX	0.392	$1.99x10^{-16a}$
	NRP1	0.455	$3.15x10^{-22a}$
Th1	*TBX21*	0.210	$1.98x10^{-5a}$
	STAT4	0.364	$2.94x10^{-14a}$
	STAT1	0.282	$6.46x10^{-9a}$
	IFN-g	0.161	0.001^a
	TNF-a	0.153	0.002^a
Th2	*GATA3*	-0.497	$7.77x10^{-27a}$
	STAT6	-0.173	$4.48x10^{-4a}$
	IL13	0.181	$2.38x10^{-4a}$
Tfh	*BCL6*	-0.144	0.004^a
	IL21	0.041	0.409
Th17	*STAT3*	0.431	$7.67x10^{-20a}$
	IL17A	-0.071	0.153

Table IV. Continued.

Cell type	Gene	Spearman's correlation	P-value
Treg	*FOXP3*	0.254	$1.91x10^{-7a}$
	CCR8	0.274	$1.80x10^{-8a}$
	TGFb	0.314	$8.82x10^{-11a}$

[a]P<0.05. TAM, tumor-associated macrophage; Th, T helper cell; Tfh, follicular helper T cell; Treg, regulatory T cell; CSF1R, colony-stimulating factor 1 receptor; CCL2, C-C motif chemokine ligand 2; IL10, interleukin 10; INOS (NOS2), nitric oxide synthase 2; IRF5, interferon regulatory factor 5; PTGS2, prostaglandin-endoperoxide synthase 2; VSIG4, V-set and immunoglobulin domain-containing 4; MS4A4A, membrane-spanning 4 domains A4A; CEACAM8, carcinoembryonic antigen-related cell adhesion molecule 8; ITGAM, integrin subunit αM; CCR7, C-C motif chemokine receptor 7; KIR2DL1, killer cell immunoglobulin-like receptor, two Ig domains and long cytoplasmic tail 1; HLA-DPB1, major histocompatibility complex class II DP β1; CD1C, CD1c molecule; ITGAX, integrin subunit αX; NRP1, neuropilin 1; TBX21, T-box 21; STAT4, signal transducer and activator of transcription 4; IFN-g, interferon γ; TNF-α, tumor necrosis factor α; GATA3, GATA-binding protein 3; BCL6, BCL6 transcription repressor; FOXP3, forkhead box P3; CCR8, C-C motif chemokine receptor 8; STAT5B, signal transducer and activator of transcription 5B; TGFB1, transforming growth factor β1.

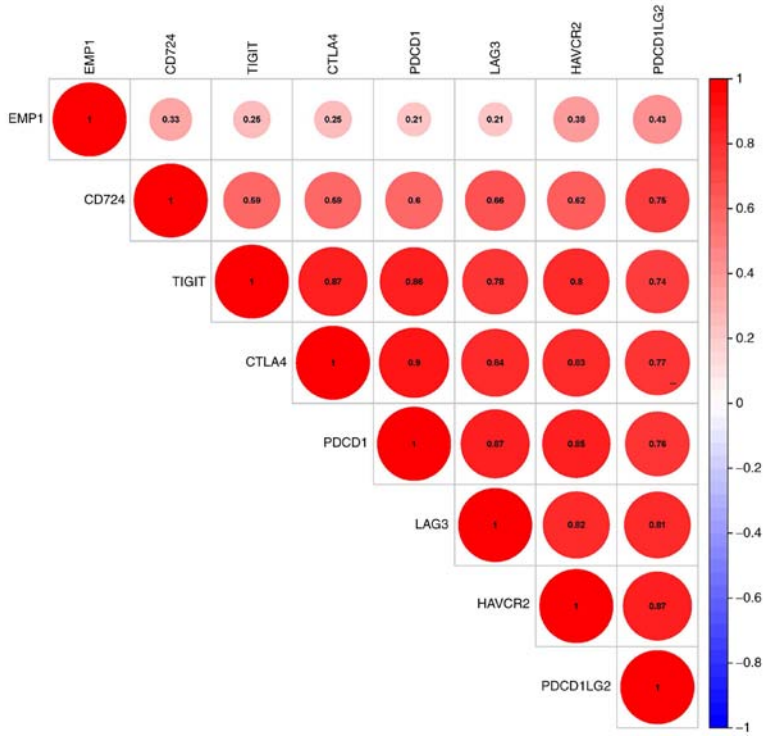

Figure 6. Correlations between EMP1 and immune checkpoint-related genes. Red represents a positive correlation, and blue represents a negative correlation. The size and color intensity of the circle indicates the strength of the correlation. The number in the circle indicates the correlation coefficient. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1.

negatively correlated with the infiltration of B cells, as well as the degree of tumor purity. Further analysis revealed that the upregulation of *EMP1* was significantly positively associated with an abundance of macrophages, but negatively associated with the levels of Tregs, plasma and CD4-naïve T cells. The co-expression analysis of *EMP1* and the previously reported immunolabeled genes also yielded consistent results. The expression level of *EMP1* in BLCA tissues was further validated using independent specimens. These results suggested that *EMP1* may be a prognostic biomarker, as well as an important factor for the recruitment and regulation of infiltrating immune cells in BLCA.

EMP1 was selected as the research object mainly based on the following considerations: Through the analysis of multiple databases, a significant difference was observed in the expression of *EMP1* between bladder cancer and paired normal tissues, and only one study suggested that *EMP1* was expressed at low levels in bladder cancer (33). However, it was necessary to further study this gene as its function in bladder cancer was not clear. *EMP1* belongs to the peripheral myelin protein 22 (*PMP22*) family and has high homology among the family members (34,35). *PMP22* is considered to serve an important role in the immune response (36-38). Thus, it was speculated that *EMP1* may exert a similar function.

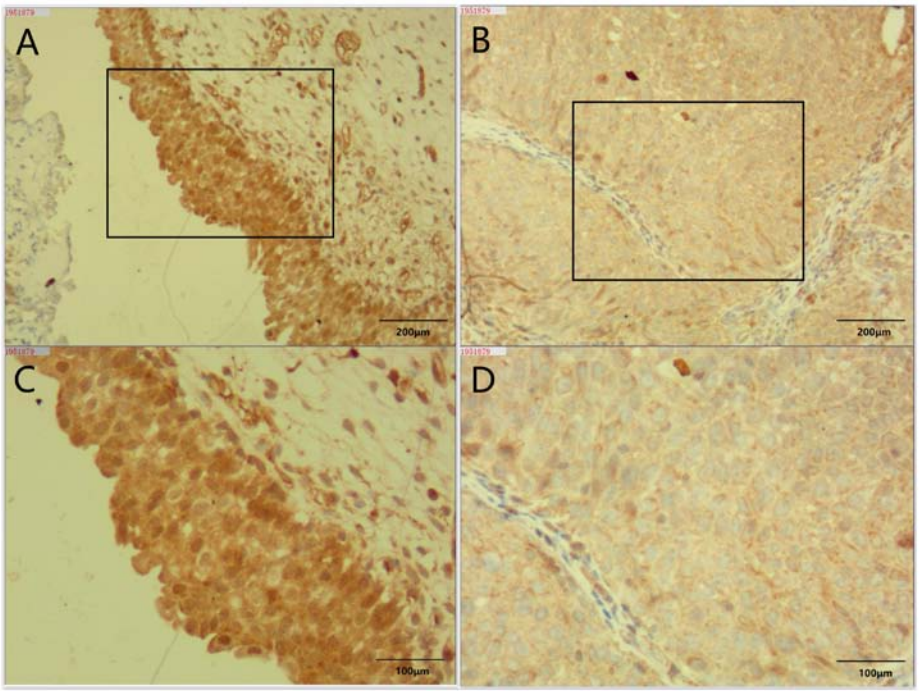

Figure 7. EMP1 is downregulated in BLCA. Immunohistochemical staining was performed to detecting the expression of EMP1 in (A and C) adjacent normal tissues and (B and D) tumor tissue specimens of patients with BLCA. (A and B) Magnification, x200. (C and D) Magnification, x400. BLCA, bladder urothelial carcinoma; EMP1, epithelial membrane protein 1.

Alteration of *EMP1* has been implicated in different types of human cancer. Cancer cell lines transfected with *EMP1 in vitro*, including PC-3 prostate cancer, SW-480 colon cancer and MCF-7 breast cancer have been demonstrated to exhibit a high rate of apoptosis and a poor survival rate (14,15). The *EMP1* gene expression has also been demonstrated to be downregulated in laryngeal, esophageal, head and neck, sclerosing gastric and prostate cancer, as well as in uterine fibroids compared with that in normal tissues (39-43). Sun *et al* (12), have demonstrated that as a tumor suppressor gene, high expression of *EMP1* can improve the 5-year survival rate of patients with nasopharyngeal cancer. This result was also confirmed in gastric cancer (13), and *EMP1* was reported to be associated with nodal metastasis in oral squamous cell cancer (44). *EMP1* overexpression has also been established to be associated with poor OS in pediatric leukemia (17).

However, some studies have yielded different results. Lai *et al* (45), demonstrated that *EMP1* expression levels were higher in non-small-cell lung cancer compared with those in the benign control group. When a recombinant adenovirus overexpressing *EMP1* was constructed and virus-infected PC9 cells were transplanted into nude mice, the growth of the transplanted tumors could be observed. Another study by Zhang *et al* (46), demonstrated that *EMP1* was upregulated in human gliomas, and that *EMP1* expression was significantly increased in patients with World Health Organization tumor grade III-IV compared with grade I-II.

The results of the present study demonstrated that *EMP1* expression was downregulated in BLCA compared with that in normal tissues, but patients with low *EMP1* expression exhibited an improved OS rate compared with those in the high expression group. These conflicting results may be caused by the function of *EMP1*. Previous studies have demonstrated that *EMP1* serves an important role in cell differentiation (35,47,48) and proliferation (34,49); thus, *EMP1* promotes cell differentiation, whereas tumor cells are characterized by de-differentiation. Low degree of tumor cell differentiation leads to low expression of *EMP1*. In addition, *EMP1* is a direct or indirect target gene of the classical proto-oncogene *c-myc*, which serves a role in promoting cell proliferation (34). The results of the present study also demonstrated that in patients with high *EMP1* expression, the prognosis of BLCA was poor. This result was contrary to that of Peter *et al* (33), whose findings suggested that low expression of *EMP1* was associated with an increased risk of urothelial cancer-specific mortality. These differences may be due to the histological differences among the tumors, due to the differences in the internal and external environments of the tumor cells, and even due to the differences in the methods of data collection and analysis.

There is relatively little information about the signaling pathways associated with *EMP1*-mediated biological processes. Silencing experiments in the T-precursor ALL and B-ALL cell lines have indicated that *EMP1* may signal through the Src kinase family (17). Wang *et al* (50), transfected *EMP1* into the esophageal cancer cell line EC9706 and reported that *EMP1* inhibited the proliferation of esophageal cancer cells, arrest the tumor cells in the S phase of the cell cycle or prolong the G1 phase. However, other study suggested that the *EMP1* gene serves a role in promoting cell proliferation as a target gene of the proto-oncogene *c-myc* (19). *EMP1* is highly expressed with *c-myc* in active embryonic stem cells, but the expression gradually decreases as the embryo differentiates and matures (51). Currently, the mechanism of *EMP1* in cell proliferation and apoptosis is not clear, but it is worth affirming that *EMP1* exerts its effects mainly by regulating signal transduction between cells or between cells

and the extracellular matrix (52). Ramnarain *et al* (21), have demonstrated that mutation in the EGFR gene leads to the activation of a series of downstream signals, including *EMP1*, as a result of which patients harboring the mutated EGFR are more likely to develop glioblastoma compared with those with wild-type EGFR. Durgan *et al* (53), have suggested that *EMP1*, as an important transcriptional target in the Ras/mitogen-activated protein kinase pathway of bronchial epithelial cells, participates in the tight junction between cells and serves an important role in tracheal morphogenesis; its deletion may be associated to lung tumors. Lai *et al* (45), have reported that high expression of *EMP1* leads to the occurrence and development of non-small-cell lung cancer by activating the PI3K/AKT pathway.

A number of previous studies have stated that members of the EMP family affect the integrin heterodimer repertoire on the plasma membrane, and modulation of the expression or localization of EMP proteins may alter the surface repertoire of molecules (54-56). The surface molecular repertoire, including major histocompatibility complex 1 proteins, integrins and other immunoglobulin superfamily members such as CD54 and glycosylphosphatidyl-inositol-linked proteins may be altered as the expression of *EMP2* changes (57). These results suggest that members of the EMP family may influence the development of cancer cells via the tumor immune microenvironment and ultimately affect the prognosis of patients. However, there is lack of research on the association between *EMP1* and different immune cell infiltration in BLCA.

Based on previous studies, the present study further analyzed the correlation between *EMP1* and the infiltration of various immune cells.

The results of the present study suggested that macrophage infiltration was an independent prognostic factor in BLCA, which was consistent with previous studies that have demonstrated significant associations between tumor-associated macrophage infiltration and shorter survival of patients with bladder cancer (10,58,59). A number of studies have reported that a high neutrophil-lymphocyte ratio is a negative predictor of bladder cancer (60-63). In addition, neutrophil infiltration is significantly associated with poor prognosis of bladder cancer (59,64). High $CD4^+$ T-cell density has been identified to be associated with poor prognosis in patients with bladder cancer (65,66), and a high level of mature tumor-infiltrating DCs predicts progression to muscle invasion in bladder cancer (58). In the present study, *EMP1* expression was positively correlated with macrophage, neutrophil, $CD4^+$ cell and DC infiltration, and negatively correlated with B lymphocyte infiltration, indicating that *EMP1* expression may be a negative regulator of tumor immunity. These results were consistent with previous studies of immune cell infiltration.

The correlation between *EMP1* expression and the enrichment of neutrophils and macrophages was further confirmed in the TISIDB in the present study. *EMP1* also exhibited a significant positive correlation with the enrichment of immune cells such as CD4, CD8, DCs, NK and NKT cells, which suggested that *EMP1* may aggravate the prognosis of patients by affecting the level of infiltration of specific immune cells. In addition, Treg and MDSC cells are considered to be suppressors of antitumor immune responses, and their enrichment is associated with poor patient outcomes in cancer (8,67-70). However, the results of B cell enrichment in TIMER and TISIBD in the present study were not consistent, reflecting the different algorithms used for the two immune scores. Although the mechanism of *EMP1* in tumor immunology is not fully understood, the correlations between *EMP1* expression and immune cell infiltration implicated the role of *EMP1* in regulating tumor immunology in BLCA.

Recent studies have provided possible mechanisms that may explain the association between *EMP1* expression and inflammation. Wang *et al* (71), have demonstrated that low microRNA-31 expression in mesenchymal stem cells in patients with psoriasis causes an increase in the expression of *EMP1*, which in turn facilitates T lymphocyte activation. A study by Pan *et al* (72) has indicated that *EMP1* is activated by zinc finger protein 750 and regulates signaling pathways associated with proliferation and inflammation in CAL-27 cells.

To further elucidate the possible mechanism of *EMP1* expression in immunity, the present study speculated its possible function by assessing co-expression with previously reported gene markers. Co-expression of *CCL-2*, *CD68*, *IL10* of TAMs, *PTGS2*, *IRF5* of M1 phenotype, *CD163*, *VSIG4* and *MS4A4A* of the M2 phenotype with *EMP1* suggested that *EMP1* may regulate macrophage polarization. DC markers (e.g., *HLA-DPB1*, *HLA-DRA*, *BDCA-1*, and *ITGAX*) also exhibited a significant correlation with *EMP1* expression, indicating an association between DC penetration and *EMP1*. Together, DC and T cells can secrete IL-12 and IL-18 to activate T cell proliferation, induce CTL production, and trigger a Th1-type immune response, which are conducive to tumor clearance (73). Treg markers (*FOXP3*, *CCR8*, *TGFB1*) were significantly co-expressed with *EMP1*. *FOXP3* serves an essential role in maintaining homeostasis of the immune system by facilitating the acquisition of full suppressive function and stability of the Treg lineage, and by directly modulating the expansion and function of conventional T cells (74). These results indicated that *EMP1* expression may serve a complex role in the immune regulation network.

Checkpoint inhibitors are monoclonal antibodies that block inhibitory checkpoint antigens and repress the stimulation of T cells, exhibiting anticancer effects (75,76). Upon chronic stimulation by tumor antigens, tumor-infiltrating T cells lose their effector functions and their ability to kill tumor cells, accompanied by a progressive increase in the diversity and number of inhibitory receptors expressed on them, including *CD274*, *PDCD1LG2*, *HAVCR2*, *CTLA4*, *LAG3*, *PDCD1* and *TIGIT* (75-82). Therefore, genes that were associated with immune checkpoints were selected for analysis in the present study.

The results of the present study revealed significant co-expression between *EMP1* and the genes reported to be associated with immune checkpoints, suggesting that *EMP1* may serve a role in BLCA by affecting these immune checkpoints. Previous results have suggested a positive correlation between *EMP1* and Treg, which inhibits the immune response of other immune cells. These results suggest that *EMP1* may restore the function of immune cells by inhibiting immune checkpoints. Blocking antibodies against *EMP1* may be a promising treatment strategy for patients with BLCA.

The present study had certain limitations. Further research, including deep sequencing, is needed to elucidate the full spectrum of variability and any functional variants of *EMP1*

in BLCA. It is also necessary to further explore the specific molecular mechanism of *EMP1* in bladder cancer cells.

In conclusion, the results of the present study demonstrated that variations in the *EMP1* expression levels were associated with the prognosis of patients with BLCA. High *EMP1* expression was associated with a poor OS rate. In addition, these results revealed that the extent of immune cell infiltration and the diversity of immune marker expression were associated with *EMP1* expression in BLCA. Therefore, the results of the present study may provide insights into the potential function of *EMP1* in tumor immunology and its potential as a cancer biomarker.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HY designed the research and reviewed the manuscript. BL analyzed the data and prepared the original draft. TZ and XY performed statistical calculation and experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Peking university Shenzhen hospital (Shenzhen, China). Signed informed consents were obtained from the patients and/or guardians.

Patient consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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