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Research article

The predictive value of PFKFB3 in bladder cancer prognosis

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ABSTRACT

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3) influences cancer progression via participating in tumor aerobic glycolysis. In this study, we aimed to evaluate the prognostic significance of PFKFB3 in bladder cancer (BLCA) patients by analyzing a combination of publicly available databases, clinical patient data, and bladder tumor samples from our hospital. Single-cell and bulk RNA-seq data of bladder cancer, obtained from ENA, GEO, and TCGA databases, were utilized for our analysis. The results indicated that PFKFB3 mRNA expression was markedly elevated in bladder cancer compared to paired normal tissue. Furthermore, BLCA patients with high PFKFB3 expression exhibited a significantly worse prognosis ($P < 0.05$). To validate these findings, clinical data and immunohistochemistry staining were performed on specimens obtained from 89 BLCA patients who underwent radical cystectomy at either Qingdao University Affiliated Hospital or Peking Union Medical College Hospital. The findings from this verification process confirmed that high expression of PFKFB3 serves as a biomarker for predicting worse prognosis in BLCA patients (OR: 2.462, 95 % CI: 1.202–5.042, $P = 0.012$). To facilitate clinical application, we developed a nomogram based on four variables, including PFKFB3 expression, to predict the survival of BLCA patients. Importantly, this nomogram demonstrated a low mean prediction error of 0.03. Taken together, our findings suggest that PFKFB3 has the potential to serve as both a prognostic biomarker and a therapeutic target for BLCA patients.

1. Introduction

Bladder cancer, a prevalent urothelial malignancy, ranks as the ninth most common cancer worldwide, accompanied by a discouraging prognosis [1]. According to the guidelines set forth by the European Association of Urology (EAU) and the National Comprehensive Cancer Network (NCCN), radical cystectomy (RC) remains the recommended initial treatment for muscle-invasive

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bladder cancer (MIBC) and high-risk non-muscle invasive bladder cancer (NMIBC) [2,3]. Unfortunately, 26.9–37.5 % patients who received operative treatment still suffered from tumor recurrence or metastasis with poor-quality life [4–6]. Consequently, it's urgent to find potential prognostic biomarkers and design advanced predictive tools for risk category assessment and prognostic assessment of BLCA patients to receive real-time individualized surveillance and personalized intervention [7,8].

Aerobic glycolysis was recognized as a common hallmark of tumor metabolism, which could help the tumor cell proliferation by generating adenosine triphosphates (ATPs) and substrates [9,10]. Additionally, aerobic glycolysis might alter infiltrated immune cells and weaken the anti-tumor immune response by fostering a tumor microenvironment characterized by lactate acidosis [11]. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) was proven as a key enzyme in the aerobic glycolysis [12]. Moreover, PFKFB3 expression has been proven up-regulated in cancer cells after oncogenes activation and tumor suppressor genes deactivation [13,14]. Many studies also demonstrated that PFKFB3 could promote growth, proliferation, migration and metastasis of cancer cells in various tumor types [15,16]. Our previous study has found that selective blockade of PFKFB3 could reduce the lactate concentration and inhibit the proliferation of bladder cancer cells in microfluidic bladder cancer tumor model [17]. However, the clinical importance and exact role of PFKFB3 in BLCA have not yet been documented.

In this study, we investigated the expression of PFKFB3 in bladder cancer and the relationship between PFKFB3 expression and BLCA prognosis via analyzing published single-cell RNA sequencing data. Subsequently, we validated PFKFB3 expression by immunohistochemistry (IHC) in BLCA patient samples from our affiliations, and explored the association between PFKFB3 expression and prognosis outcomes of BLCA patients. Finally, a prognostic nomogram based on PFKFB3 and other predictive factors was designed to illustrate the role of PFKFB3 in BLCA patients' prognosis.

2. Materials and methods

2.1. Gene expression profile data and pre-processing of raw single-cell sequencing data

The single cell RNA sequencing data of eight bladder cancer tissue (4 MIBC tissue and 4 NMIBC tissue) and three normal bladder tissue were download from European Nucleotide Archive (ENA) database (website: https://www.ebi.ac.uk/ena/, and the accession ID: PRJNA662018) [18] and Gene Expression Omnibus (website: https://www. ncbi.nlm.nih.gov/geo/, and the accession ID: GSE145140) [19]. The bulk mRNA sequencing data and clinical information of The Cancer Genome Atlas (TCGA) bladder cancer cohorts were obtained from TCGA database (https://tcga-data.nci.nih.gov/tcga/). To process the raw sequencing data, we utilized the Cell Ranger toolkit (version 2.1.1) for mapping the data to the human genome (GRCh38 Ensemble build 92), generating a gene expression matrix. Subsequently, the UMI count data from all samples were imported into the Seurat package (v3.2.2) in R software (v4.2.1) for further analysis.

Cells which expressed 200 to 2500 genes and had less than 10 percent mitochondrial gene content were screened for further analysis [20]. Each UMI count matrix was scaled by library size and log-transformed. The top 3000 highly variable genes were identified through the function "FindVariableGenes" in Seurat package [21]. Then all the datasets were integrated using the "FindIntegrationAnchors" and "IntegrateData" functions in Seurat package [22].

Clustering and cell-type annotation in bladder cancer tissues and normal bladder tissue.

Following the pre-processing of the downloaded data, we performed data clustering and cell-type annotation to figure out the different cell clusters in bladder cancer tissues and normal bladder tissue. The dimensionality-reduced data were visualized by the UMAP algorithm embedded in the Seurat package [23]. The clustering analysis was carried out by using the "FindClusters" function of the Seurat package. The "FindAllMarkers" function was utilized to pinpoint specific markers within each cluster, and clusters were classified into known cell types based on canonical markers specific to each cell type [24]..

Differentially expressed genes (DEGs) screening and gene-set enrichment analysis in bladder cancer tissues and normal bladder tissue.

We identified differentially expressed genes (DEGs) between bladder cancer cells and paired normal tissue by utilizing the Seurat package "FindMarkers" function. The following parameters were used: PCT (Percent of positive expression in epithelial cells) > 0.1, adjusted P value < 0.01, and log2|Fold Change|>0.15. The differential expression of PFKFB3 between bladder cancer cells and normal bladder epithelial cells was displayed by using t-SNE plot. The DEGs were then submitted to the Metascape software (http://metascape.org/gp/index.html#/main/step1) for further gene-set enrichment analysis.

2.2. Patients and samples

Between October 2010 and March 2015, 89 patients who diagnosed pathologically as muscle invasive bladder cancer (MIBC) or high-risk non-muscle invasive bladder cancer (NMIBC) at either Qingdao University Affiliated Hospital or Peking Union Medical College Hospital were included in this study. The diagnosis of urothelial transitional cell carcinoma was validated by pathologic examination of tumor specimens [25]. All 89 patients, whether primary or recurrent cases, underwent radical cystectomy (RC) at our hospitals. Bladder tumor samples were collected and preserved by fixing them with formalin and embedding them in paraffin for standard pathological examination. The follow-up period for bladder cancer patients ranged from 3 to 90 months. Exclusion criteria included (1) Patients with bladder tumors of other histological types, such as adenocarcinoma and squamous cell carcinoma; (2) Patients lacking sufficient tumor tissues for additional analysis; (3) Patients with a prior diagnosis of another tumor type which can affect the prognosis of patients. All participants included in the study provided informed consent.

2.3. Immunohistochemistry

We sliced the tissues, which were fixed in formalin and embedded in paraffin, into 4 μm sections. Ethanol was used to deparaffinize the sections. Sections were heated to 121 °C in 1 mmol/L EDTA, cooled to 90 °C, incubated for 5 min, and then treated with peroxidase blocking reagent (Dako, Denmark) to retrieve antigens. Next, the sections underwent a 10-min incubation at room temperature with a 1:50 dilution of polyclonal rabbit *anti*-PFKFB3 primary antibody (Abcam, Cat. ab181861, USA), followed by a 2-h incubation with

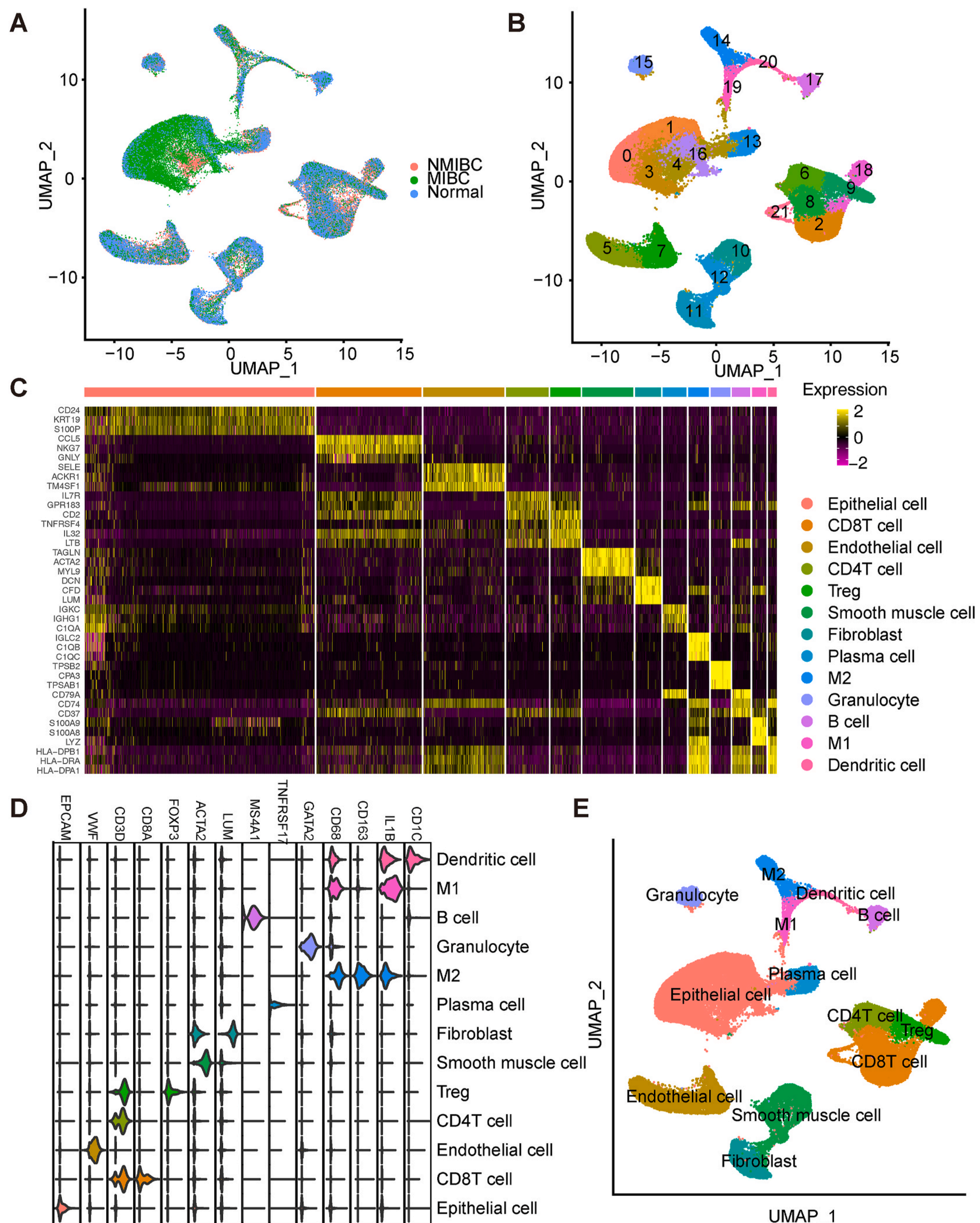

Fig. 1. Identification of bladder cancer cells in single-cell sequencing data. A. UMAP plot showing the integrated bladder cancer single cell data. Each point represents a cell. Points were colored according to the pathological tissue type. B. UMAP plot showing the cell clusters identified by the clustering algorithm. Points were colored according to the cluster identity. C. Heatmap showing the automatically identified markers for each cell type. D. Violin plot showing the expression of classical markers of each cell type. E. UMAP plot showing the annotated cell clusters. Each point represents a cell. Points were colored according to the cell type.

mouse anti-rabbit HRP-labeled antibody (Solarbio, China). Subsequently, they were stained by DAB Horseradish Peroxidase Color Development Kits (Beyotime, China) for 30 min and lightly counterstained with hematoxylin.

Two independent pathologists, who were unaware of the clinical information of the enrolled patients, evaluated the stained sections. Each section received a score based on staining intensity and the proportion of positive cells [26,27].: Sections with no staining were scored by 0 point; sections with light yellow staining were scored by 1 point; sections with light brown staining were scored by 2

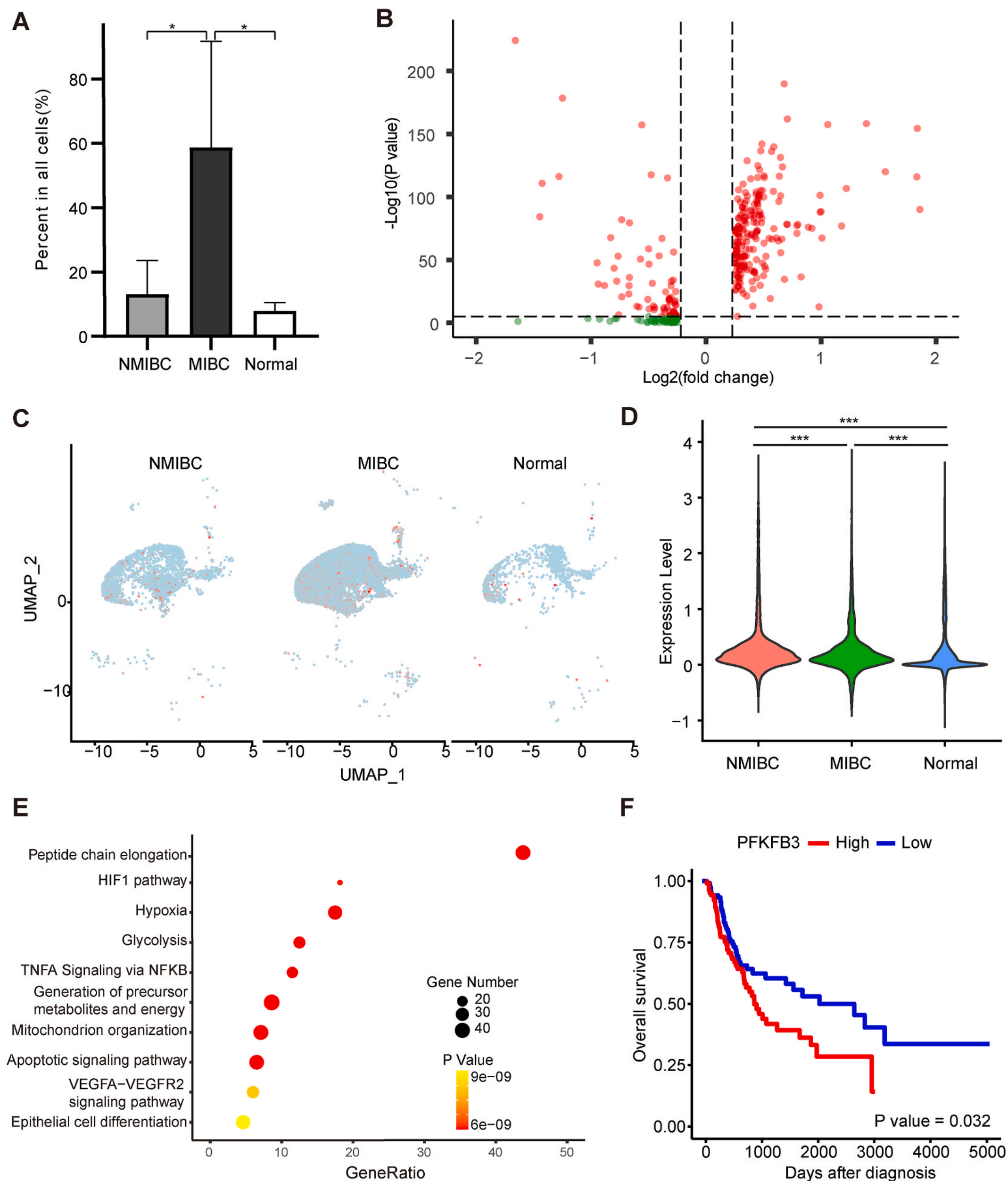

Fig. 2. Alteration of PFKFB3 in bladder cancer cells. A. Histogram showing the percent of EPCAM-positive epithelial cells in NMIBC, MIBC and normal bladder mucosa. *: $P < 0.05$ B. Volcano plot showing the DEGs between cancer cells and normal epithelial cells. Blue points represent genes satisfy the P value threshold, and the red points represent genes satisfy both the P value and fold change threshold. C. Scatter plot showing the expression of PFKFB3 in epithelial cells in NMIBC, MIBC and normal bladder mucosa. Red points represent cells with positive PFKFB3 expression. D. Violin plot showing the expression of PFKFB3 in epithelial cells of NMIBC, MIBC and normal bladder mucosa. ***: $P < 0.001$. E. Gene set enrichment analysis of the up-regulated DEGs. The y-axis shows significantly enriched GO terms, and the x-axis shows. Gene count refers to the number of DEGs enriched in a GO or KEGG term. Gene ratio refers to the ratio of the number of DEGs enriched in a gene set to the total number of genes in the gene set. F. Kaplan-Meier survival analysis showed that bladder cancer with high PFKFB3 expression showed significant poor overall survival ($P = 0.032$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

points; and sections with dark brown staining were scored by 3 points; Sections with less than 5 % positive cells were scored by 0 point; sections with 6–25 % positive cells were scored by 1 point; sections with 26–50 % positive cells were scored by 2 points; sections with 51–75 % positive cells were scored by 3 points; and sections with more than 75 % positive cells were scored by 4 points. The final score of PFKFB3 expression was calculated by multiplying the staining intensity scores and the positive cell ratio scores. Sections with more than 6 scores were defined as high PFKFB3 expression, otherwise low PFKFB3 expression.

2.4. *Statistical analysis*

Statistical analysis was performed by using SPSS software (version 24.0). Numeric data were analyzed by using Student's *t*-test or one-way analysis of variance (ANOVA). Binary data were analyzed by using chi square test. The Kaplan-Meier (KM) method and log rank test were used to analyze the influence of PFKFB3 expression on overall survival (OS). Univariate Cox regression model was used to estimate the prognostic significance of each clinical characteristics and PFKFB3 expression. Variables which showed $P < 0.05$ in the univariate analysis were included in multivariate Cox regression analysis. Variables which showed $P < 0.1$ in the multivariate analysis were regarded as prognostic factors for survival outcome. Package *nomogramEx* was used to draw a nomogram to facilitate the use of this multivariate Cox regression model. Package *rms* of R software (Version 4.2.1) was used to validate the nomogram. Fifty samples were randomly sampled from the whole cohort to build a Cox 3-year survival predicting model. And the left patients were used to validate the Cox model. The validation was performed for 1000 time, and the average predicted survival was compared with the observed survival.

3. Results

3.1. *Clustering and annotation of bladder cancer cells and paired normal bladder cells by analyzing single-cell RNA sequencing data*

A total of 65,723 filtered cells underwent comprehensive bioinformatic analysis, comprising 49869 bladder cancer cells (25576 cells from NMIBC, 24320 cells from MIBC) and 15827 paired normal bladder cells (Fig. 1A). Through meticulous clustering analysis, we successfully identified and delineated 22 distinct cell clusters (Fig. 1B) Subsequently, each cell cluster was meticulously annotated based on the automatic identification of cell-type specific markers (Fig. 1C) and canonical cell-type markers (Fig. 1D). Epithelial cells were primarily distinguished by elevated EPCAM expression. Endothelial cells were characterized by the presence of VWF expression. T cell subtypes were marked by CD3D, CD8A, and FOXP3 expression. Smooth muscle cells, also known as cancer associated fibroblasts, were marked by high ACTA2 expression. Fibroblasts were identified by LUM expression. B cells and plasma cells were recognized by MS4A1 and TNFRSF17 expression, respectively. Granulocytes were distinguished by GATA2 expression, while various macrophage subtypes were classified based on CD68, CD163, IL1B, and CD1C expression (Fig. 1D). In total, 13 distinct cell types were identified, including epithelial cells, CD4T cells, CD8T cells, Treg cells, Endothelial cells, Smooth muscle cells, Fibroblasts, Plasma cells, M1 macrophages, M2 macrophages, Dendritic cells, Granulocytes, and B cells (Fig. 1E). It is notable that the maximal number of epithelial cells was observed within the analyzed samples, showcasing their predominant presence.

3.2. *Changes of epithelial cells and differential expressed genes (DGEs) between bladder cancer and normal bladder cells*

Subsequently, proportional analysis revealed that epithelial cells accounted for 7.87 % in normal bladder mucosa, 13.53 % in NMIBC, and a significantly higher proportion of 73.20 % in MIBC. Notably, a gradual increase in epithelial cells was observed, exhibiting a statistically significant trend among normal bladder mucosa, NMIBC, and MIBC (ANOVA $P = 0.019$, Fig. 2A). Furthermore, 622 genes exhibited differential expression between bladder cancer cells and normal bladder mucosal epithelial cells. (Fig. 2B). Among these DEGs, 447 genes, including PFKFB3, were found to be over-expressed in bladder cancer cells. Remarkably, the expression of PFKFB3 in epithelial cells was markedly higher in MIBC compared to NMIBC (Fig. 2C and D). Gene set enrichment analysis revealed that upregulated differentially expressed genes in cancerous epithelial cells were mainly enriched in glycolysis pathway, HIF1 pathway, apoptotic signaling pathway, and epithelial cell differentiation pathway, and more (Fig. 2E). To investigate the potential impact of PFKFB3 mRNA expression in BLCA, we retrieved bulk transcriptomic sequencing data and clinical information from the TCGA database for 234 primary MIBC patients. Kaplan-Meier survival analysis showed that MIBC patients with high PFKFB3 mRNA expression had significant poor overall survival compared with the patients with low PFKFB3 mRNA expression ($P = 0.032$, Fig. 2F).

3.3. *Clinical characteristics of patients*

To further confirm the elevated expression of PFKFB3 in bladder cancer patients with different clinical characteristics and assess its prognostic significance in BLCA, we included a retrospective cohort of 89 consecutive bladder cancer patients from our hospital. Among these patients, 76 (85.39 %) were males and 13 (14.61 %) were females. The mean age of the cohort was 69.1y, with 50 (56.18 %) individuals having a smoking history. Of the enrolled patients, 72 (80.90 %) of them was diagnosed as MBIC, while the rest 17 (19.10 %) as NMIBC. Notably, 10 (11.24 %) patients had previously undergone Bacillus Calmette-Guerin vaccine or intravesical pirarubicin therapy. 59 (66.29 %) had a single tumor focus, while 30 (33.71 %) had multiple tumors. On average, each individual had 1.9 tumors, with the largest tumor averaging 4.3 cm in diameter. Pathologically, 76 (85.39 %) tumors exhibited low differentiation, whereas 13 (14.61 %) showed high differentiation. Vessel or nerve invasion was observed in tumors from 11 (12.36 %) and 8 (8.99 %) patients, respectively. The median follow-up duration was 33.2 months (Table 1).

3.4. PFKFB3 expressions and associations between PFKFB3 expressions and clinical characteristics in bladder cancer patients

After confirming the elevated expression of PFKFB3 through the analysis of public data, we proceeded to validate its increased expression in bladder cancer tissue using immunohistochemistry. The results revealed predominant nuclear staining of PFKFB3 in cancer cells, with scarce cytoplasmic staining observed (Fig. 3). 50 (56.18 %) tumor tissues showed high PFKFB3 expression, and 39 (43.82 %) tumors showed low PFKFB3 expression. Subsequently, we conducted an analysis to explore the association between PFKFB3 expression and various clinical characteristics in bladder cancer patients to determine whether they impacted on the expression of PFKFB3 expression. Results indicated that PFKFB3 expression did not significantly differ in gender, smoking history, pathological characteristics, intravesical therapy, tumor diameter or numbers. (Table 2).

3.5. Prognostic significance of PFKFB3 expression

The elevated expression of PFKFB3 in bladder cancer has been confirmed, and there is no association between PFKFB3 expression and clinical characteristics of bladder cancer patients. Subsequently, we conducted a further analysis to examine the relationship between PFKFB3 expression and overall survival (OS) in bladder cancer patients. KM analysis revealed that high PFKFB3 expression correlated with poorer overall survival (OS) in BLCA patients post-cystectomy ($P = 0.017$, Fig. 4A). Further, we tried to find out the predictive factors in bladder cancer prognosis. And results showed that high PFKFB3 expression (OR: 2.172, 95 % CI: 1.115–4.232, $P = 0.023$) and nerve infiltration (OR: 2.801, 95 % CI: 1.153–6.804, $P = 0.023$) significantly negatively impacted the overall survival (OS) of BLCA patients in univariate Cox regression analysis (Table 3). Furthermore, in the multivariate Cox regression model, both high PFKFB3 expression (OR: 2.462, 95 % CI: 1.202–5.042, $P = 0.012$) and nerve invasion (OR: 2.986, 95 % CI: 1.228–7.261, $P = 0.016$) emerged as prognostic indicators for postoperative OS in BLCA patients (Table 3). Finally, we used *nomogramEx* package and the *rms* package to build and verify a nomogram. Results indicated that BLCA patient scoring was based on age (0–95 scores), presence of MIBC (44 scores for MIBC and 0 score for NMIBC), PFKFB3 expression (0 score for low PFKFB3 expression and 41.5 scores for high PFKFB3 expression), and nerve infiltration (0 score for no nerve infiltration and 62.5 scores for nerve infiltration). The total points are calculated by adding these scores, and the number directly below the accumulated total points represents the predicted 3-year survival for the patient. (Fig. 4B). Results showed that the survival predicted by the COX model was well similar to the observed survival (Mean error = 0.03, Fig. 4C).

4. Discussion

The reprogramming of energy metabolism has been widely observed in most cancer cells. Otto Warburg et al. found that most cancer cells tended to rely on glycolysis for generating ATPs even in an aerobic environment, which was called as the Warburg effect [28]. In most cancer cells, modifications in the protein expression profiles was used to adjust to the altered energy metabolism, and glycolysis-related proteins were over-expressed to facilitate the influx and utilization of glucose [29]. This up-regulation of glycolysis-related proteins is also observed in bladder cancer tissues, suggesting that the Warburg effect may contribute to tumor initiation and progression in patients with bladder cancer [30].

Research has demonstrated that the initial key step of glycolysis is the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6P2) by the enzyme 6-phosphofructo-1-kinase (PFK-1) [12]. PFK-1 was allosterically regulated by several

Table 1
Baseline information of enrolled bladder cancer patients.

Gender	Male	76
	Female	13
Age (year)		69.1 ± 10.6
Differentiation	Low	76
	High	13
Muscle infiltration	No	17
	Yes	72
Vessel invasion	No	78
	Yes	11
Nerve invasion	No	81
	Yes	8
History of smoking	No	39
	Yes	50
History of TURBT[a]	No	79
	Yes	10
Intravesical therapy	No	79
	Yes	10
Maximum tumor diameter (cm)		4.3 ± 1.9
Number of tumors		1.9 ± 1.5
Follow-up time (month)		33.2 ± 19.4

[a] TURBT:Transurethral resection of bladder tumor.

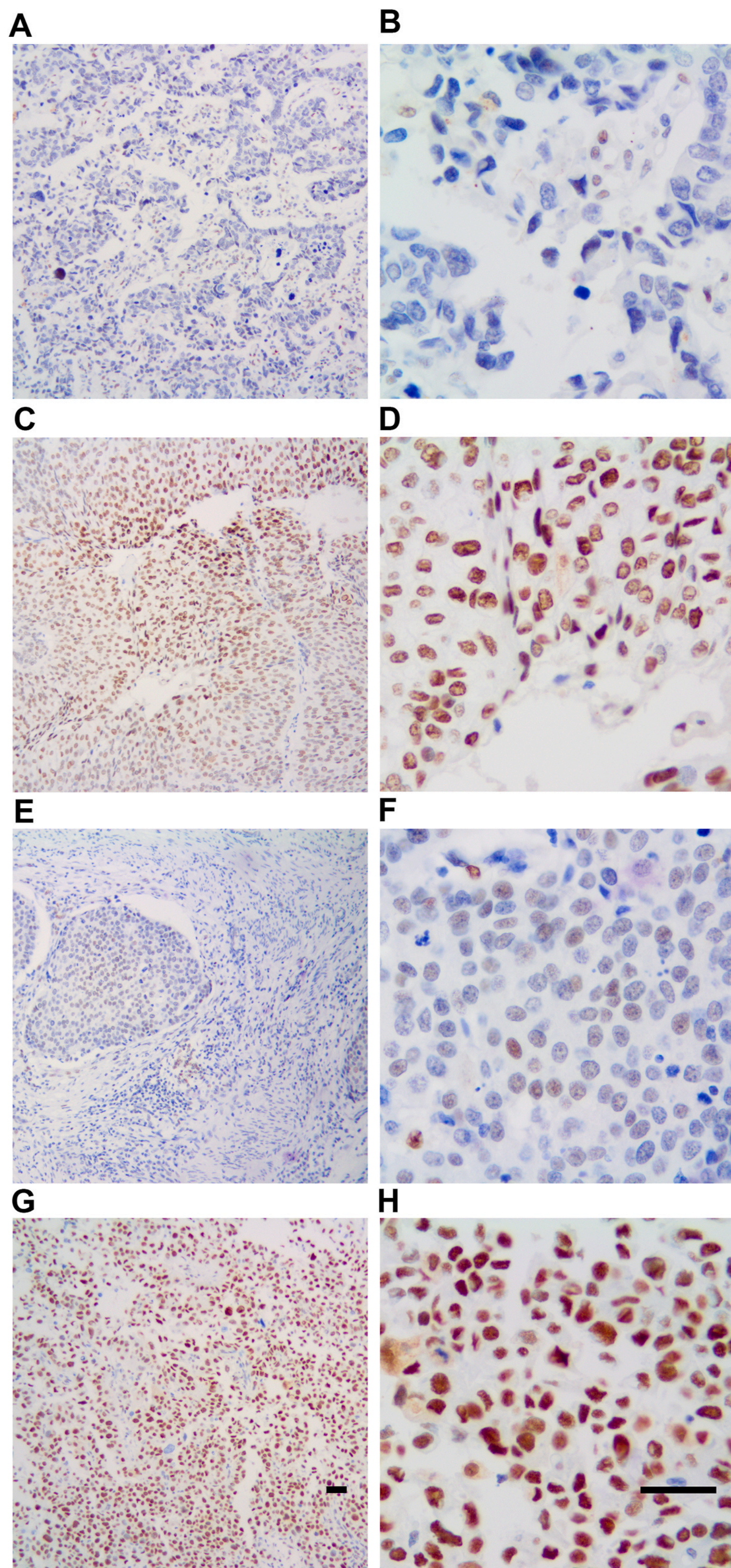

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Fig. 3. Expression of PFKFB3 protein in bladder cancer tissue. A and B: Low PFKFB3 expression in NMIBC. C and D: High PFKFB3 expression in NMIBC. E and F: Low PFKFB3 expression in MIBC. G and H: High PFKFB3 expression in MIBC. Magnification: A, C, E and G: 100×; B, D, F and H: 400X. Scale bar: 10 μm.

Table 2
The relationship between PFKFB3 expressions and clinical characteristics in bladder cancer patients.

PFKFB3 expression		Low	High	P value
Gender	Male	46	30	0.124
	Female	11	2	
Differentiation	Low	46	30	0.124
	High	11	2	
T Stage	T1	12	5	0.503
	T2a	19	8	
	T2b	22	18	
	T3	2	1	
	T4	2	0	
Muscle infiltration	No	12	5	0.532
	Yes	45	27	
Vessel invasion	No	49	29	0.740
	Yes	8	3	
Nerve invasion	No	51	30	0.706
	Yes	6	2	
History of smoking	No	29	10	0.073
	Yes	28	22	
Intravesical therapy	No	51	28	0.742
	Yes	6	4	
Maximum tumor diameter (cm)		4.35 ± 2.05	4.06 ± 1.53	0.484
Number of tumors		2.04 ± 1.58	1.72 ± 1.40	0.348
Age		67.49 ± 11.01	71.94 ± 9.32	0.060

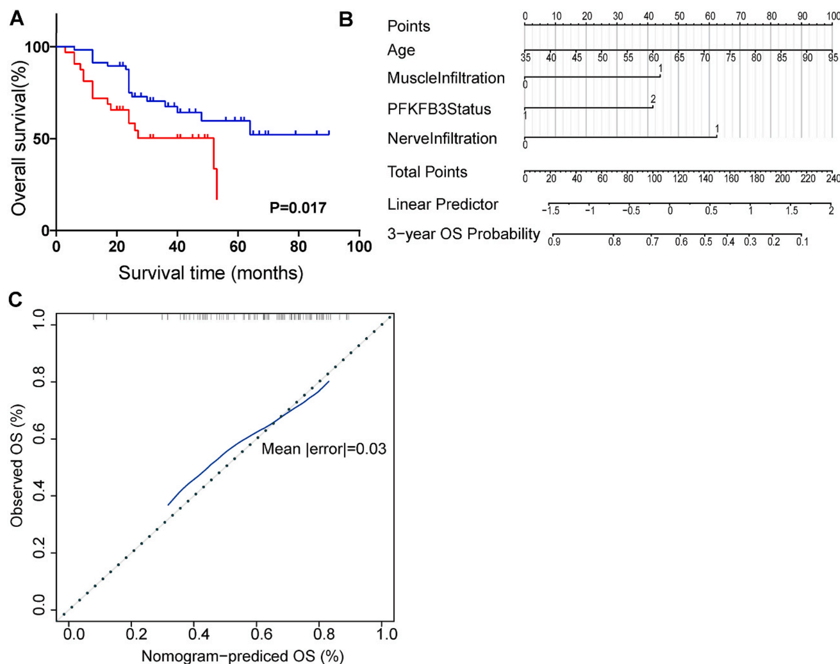

Fig. 4. Influence of PFKFB3 protein expression on the OS bladder cancer patients. A. Kaplan-Meier analysis survival analysis of PFKFB3 expressions in bladder cancer. High PFKFB3 patients showed significant poor overall survival in all bladder cancer patients (log-rank test, P = 0.017). B. Nomogram for the prediction of OS of bladder cancer patients. C. Calibration plot of the nomogram. X axis represents the OS predicted by nomogram, while the Y axis represents the observed OS. The observed OS was well represented by the predicted OS, with a mean absolute error of 3 %.

Table 3
Univariate and multivariate Cox regression of predictive factors in bladder cancer prognosis.

	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P value	OR (95%CI)	P value
Age	1.031 (0.997–1.067)	0.074	1.025(0.989,1.063)	0.177
Gender	2.344 (0.718–7.655)	0.158		
Tumor Size	1.065 (0.916–1.238)	0.412		
Tumor Number	0.858 (0.669–1.100)	0.228		
Muscle Infiltration	0.399(0.14–1.135)	0.085	0.505(0.173,1.473)	0.211
Vessel invasion	1.547 (0.642–3.729)	0.331		
Nerve invasion	2.801 (1.153–6.804)	0.023*	2.986 (1.228,7.261)	0.016*
History of Smoking	0.91(0.473–1.753)	0.779		
Intravesical therapy	1.438(0.558–3.706)	0.451		
PFKFB3 expression	2.172 (1.115–4.232)	0.022*	2.462 (1.202–5.042)	0.012*

metabolites, including ATP and fructose 2,6-bisphosphate (F-2,6-BP), which was a shunt product converted from F6P by PFK2 [31]. There were four PFK2 isozymes in human, and the PFKFB3 played the most important role in phosphatase activity ratio, which could sustain high glycolysis rates [32]. More importantly, the expression of PFKFB3 in nucleus could promote cell growth by upregulating CDK1, CDC25C, CCND3, and downregulating p27 protein levels, which might be a novel drug target in tumor treatment [33]. The overexpression of PFKFB3 was found in different cancer types, such as breast cancer, ovarian cancer, liver cancer, etc [34–36]. For example, PFKFB3 could promote cell proliferation by increasing the expression of several key cell cycle-related proteins, including CDK1, Cdc25C, and cyclin D3, and decreasing the expression of the cell cycle inhibitor p27 in hepatocellular carcinoma and HeLa cells [37]. Furthermore, glycolysis exerts a profound influence on the tumor microenvironment (TME), which has been proven to impact malignant behavior, disease progression, therapeutic responses, and clinical outcomes [38]. The intricate microenvironment within cancerous tissue has also been identified as a critical factor affecting the utilization of glycolysis for energy metabolism [39]. Previous studies also demonstrated that various mesenchymal cells and immune cells in TME took part in the process of glycolysis in bladder cancer tissues [40,41].

However, there is a lack of studies specifically focusing on the impact of PFKFB3 overexpression on BLCA prognosis. Our study found that the proportion of mesenchymal and immune cells reached 86.47 % and 26.8 % in all NMIBC and MIBC cells. The increased expression of PFKFB3 in cancer cells might be masked by other cells with glycolytic metabolism, like immune cells, in bulk RNA sequencing and Western blot assays, which might cause the scientists ignore the potential influence of PFKFB3 in BLCA. Single-cell sequencing, which could help in analyzing the biological characteristics and dynamics of cancers, might be an effective technique to figure out the potential effect of PFKFB3 in BLCA [42]. Therefore, we explored single-cell RNA sequencing data from bladder cancer cells for uncovering the heightened glycolytic activity in malignant epithelial cells of bladder cancer at the individual cell level.

In our study, we initially utilized single-cell RNA sequencing data to provide evidence for the elevated expression of PFKFB3 in BLCA tissues, and the relationship between PFKFB3 expression and BLCA prognosis. Subsequently, immunohistochemistry (IHC) staining confirmed PFKFB3 expression of bladder cancer via samples obtained from our hospital. The IHC staining revealed that PFKFB3 was predominantly localized in the nuclei of cancer cells, suggesting a significant positive effect on cell proliferation [31]. Our analysis of clinical follow-up data indicated a correlation between high PFKFB3 expression and poor OS in BLCA patients. This association between PFKFB3 expression and OS remained significant in both univariate and multivariate COX regression analyses. These findings indicate that PFKFB3 holds promise as a prognostic biomarker for BLCA patients. Moreover, we built a visualizable nomogram to predict the 3-year overall survival of BLCA patients to facilitate the clinical use of PFKFB3. Scores of patients' age, existence of MIBC, and PFKFB3 expression status were accumulated to predict the 3-year-survival probability of each BLCA patients. And a calibration plot analysis was used to validate the accuracy of this nomogram, and we found that the nomogram was well fitted to the observed 3-year OS.

However, there are some limitations in this study. First, the microarray and sequencing data from public database lacked granularity and specificity, which might cause systematic bias, but the analysis of clinical samples from different hospital in our study might reduce its negative effect. Second, the absence of quantitative PCR (qPCR) data and in vivo/in vitro experiments to validate our findings on the potential role of PFKFB3, as well as direct evidence of how PFKFB3 influences BLCA prognosis, is notable. Additionally, the retrospective collection of patient data in our hospital may introduce bias. Conducting systematic subgroup analyses to explore the influence of PFKFB3 on prognosis could reduce the risk of bias. Lastly, the lack of specific *anti*-PFKFB3 monoclonal antibodies hinders our ability to gather detailed clinical data regarding the effects ofanti-PFKFB3 targeting drugs on inhibiting tumor growth and improving survival in BLCA patients. Nevertheless, our study highlighted the differential expression of PFKFB3 in bladder cancer cells and its strong correlation with BLCA prognosis.

5. Conclusion

In conclusion, our study demonstrates a significant elevation of PFKFB3 expression in bladder cancer cells compared to normal tissue. Importantly, bladder cancer patients with high levels of PFKFB3 protein expression exhibit significantly worse survival outcomes. To enhance the clinical application of PFKFB3, we constructed a nomogram based on PFKFB3 expression, age, nerve invasion, and muscle infiltration to estimate overall survival probability. Nonetheless, further investigations are required to elucidate the

PFKFB3 prognostic impact in different stages and pathological types of bladder cancer, and the underlying mechanisms through which PFKFB3 expression impacts prognosis, which may help to clarify the role of PFKFB3 and provide guidance for follow-up and treatment of bladder cancer patients.

Ethics approval and consent to participate

This study was approved by the ethics committee of Peking Union College Hospital (I-20PJ0903) and Affiliated Hospital of Qingdao University (D20QDA1H032). Written informed consent was obtained from all individual participants included in the study.

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Consent for publication

Not applicable.

Data availability statement

The single-cell sequencing data in this study can be downloaded from the ENA database (https://www.ebi.ac.uk/ena/, accession ID: PRJNA662018) and Gene Expression Omnibus database (https://www. ncbi.nlm.nih.gov/geo/, accession ID: GSE145140). The bulk mRNA sequencing data and clinical information of TCGA bladder cancer cohorts could be downloaded in TCGA database (https://tcga-data.nci.nih.gov/tcga/). Other data and codes used in the current study is available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Dongxu Qiu: Writing – review & editing, Writing – original draft, Validation, Software, Formal analysis. **Bin Zhao:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Wenda Wang:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Guoyang Zheng:** Visualization, Validation, Software, Resources. **Zhan Wang:** Software, Resources, Methodology, Investigation. **Xu Wang:** Visualization, Software, Data curation, Conceptualization. **Yanan Li:** Visualization, Validation, Supervision, Software, Methodology. **Zhangcheng Liao:** Software, Methodology, Investigation, Formal analysis, Data curation. **Yang Zhao:** Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Yushi Zhang:** Validation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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