


## Identification and validation of poor prognosis immunoevasive subtype of muscle-invasive bladder cancer with tumor-infiltrating podoplanin<sup>+</sup> cell abundance

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### ABSTRACT

The choice of chemo- or immuno-therapy for muscle-invasive bladder cancer (MIBC) patients remains contentious. Podoplanin is newly identified as an immune checkpoint which intrigues us to explore the clinical significance and immunoregulatory role of tumor-infiltrating podoplanin<sup>+</sup> cells (PDPN<sup>+</sup> cells) in MIBC. A retrospective analysis of 259 MIBC patients from Zhongshan Hospital (n = 141) and Shanghai Cancer Center (n = 118) was conducted. A total of 406 MIBC patients from TCGA database were enrolled to investigate the relationship between PDPN and molecular characterization. We found that tumor-infiltrating PDPN<sup>+</sup> cell abundance indicated an inferior overall survival and recurrence-free survival. pT2 MIBC patients with PDPN<sup>+</sup> cell low infiltration could benefit more from adjuvant chemotherapy (ACT). Increased PDPN<sup>+</sup> cell infiltration was associated with diminished GZMB and TNF- $\alpha$  expression while correlated with expanded PD-1, PD-L1, LAG-3 and TIM-3 expression and tumor-promoting regulatory T cell and M2 macrophage infiltration. Tumors with high PDPN mRNA expression mainly presented luminal-infiltrated and basal-squamous subtypes (2017 TCGA classification) or stroma-rich and Ba/Sq subtypes (consensus classification). Elevated PDPN mRNA expression was associated with less FGFR3 activation signature and more T-cell-inflamed signature and EGFR activation signature. In conclusion, tumor-infiltrating PDPN<sup>+</sup> cells could be applied as an independent prognosticator for clinical outcome and a predictive biomarker for suboptimal ACT responsiveness, which was also associated with immunosuppressive contexture infiltration. Intratumoral PDPN expression had a correlation with MIBC molecular classification and therapy-related signatures. The novel immune checkpoint PDPN should be considered as a possible immunotherapeutic target for MIBC.

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muscle-invasive bladder cancer; podoplanin; tumor microenvironment; adjuvant chemotherapy; molecular subtype








## Introduction

Bladder cancer is a prevalent disease with substantial mortality rate and recurrence risk. Roughly 25% of patients are diagnosed as muscle-invasive or metastatic disease with inferior prognosis.<sup>1</sup> Adjuvant cisplatin-based chemotherapy is applied for post-operative muscle-invasive bladder cancer (MIBC) patients to improve survival outcomes.<sup>2</sup> However, several meta-analyses illustrated that adjuvant chemotherapy (ACT) could only reduce a quarter risk of death.<sup>3</sup> Chemotherapeutic resistance limited the clinical application of ACT. Further classification of MIBC that can predict survival outcomes and chemotherapeutic responsiveness is eagerly needed.

Beyond cytotoxic chemotherapy, an expanded range of effective therapies for MIBC are evolving in recent years.<sup>4,5</sup> Targeted therapies are exhibiting promising results in MIBC management. BLC2001 trial showed an impressive tumor response rate with the use of erdafitinib, a tyrosine kinase inhibitor of fibroblast growth factor receptor (FGFR) 1–4.<sup>6</sup>

Targeting epidermal growth factor receptor (EGFR) showed no significant improvements in outcome, which need further exploration with suitable biomarkers or mechanisms.<sup>7</sup> Meanwhile, immune checkpoint (ICK) blockade therapy such as nivolumab (anti-PD-1) and atezolizumab (anti-PD-L1) promotes MIBC treatment significantly as shown in CheckMate 275 trial and IMvigor 210 trial.<sup>8,9</sup> Advancing molecular pathology has reclassified MIBC subgroups to potentially support the choice of ACT, targeted therapy or ICK blockade therapy.<sup>10,11</sup> Predictive biomarkers for individualized cancer therapies are still being pursued.

Podoplanin (PDPN) is a mucin-type transmembrane glycoprotein, which is a specific marker for lymphatic endothelial cells<sup>12</sup> and also expressed on various immune cells.<sup>13</sup> Previous studies uncovered that PDPN<sup>+</sup> macrophages promoted lymphangiogenesis and lymphoinvasion in breast cancer<sup>14</sup> while PDPN<sup>+</sup> myeloid cells suppressed T cell infiltration in glioma.<sup>15</sup> Moreover, PDPN serves as a marker for nonpathogenic Th17

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subset, negatively regulating Th17-mediated inflammation.<sup>16</sup> Recently, PDPN has been identified as a novel ICK expressing on CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells, which limits the survival of T cells and orchestrates an exhausted T cell phenotype.<sup>17</sup> As a newly-discovered ICK, PDPN is a potential immunotherapeutic target. However, the clinical significance of PDPN<sup>+</sup> cells infiltrating in tumors still remains ambiguous. The role of PDPN in regulating the tumor immune microenvironment requires more elucidation.

Herein, our results indicated that tumor-infiltrating PDPN<sup>+</sup> cell abundance identified a poor prognosis subset of MIBC patients with suboptimal ACT responsiveness and immunosuppressive contexture infiltration. Intratumoral PDPN expression was associated with MIBC molecular classification and signatures of PD-L1 blockade immunotherapy and FGFR- and EGFR-targeted therapy. Targeting PDPN could be a potential therapeutic approach for MIBC.

## Materials and methods

### Ethics approval and consent to participate

Written informed consent was obtained from each patient enrolled in this study. The study was conducted and performed after approved by the Clinical Research Ethics Committee of Zhongshan Hospital and the Ethics Committee of Fudan University Shanghai Cancer Center.

### Study population

A total of 393 bladder cancer patients were enrolled who underwent radical cystectomy at Zhongshan Hospital of Fudan University from 2002 to 2014 ( $n = 215$ , Zhongshan Cohort) and Fudan University Shanghai Cancer Center from 2008 to 2012 ( $n = 178$ , FUSCC Cohort). The tissue samples of Zhongshan Cohort and FUSCC Cohort were formalin-fixed and paraffin-embedded. The enrolled cases conformed the following inclusion criteria: (a) informed consent; (b) performed radical cystectomy for bladder cancer; (c) available for paraffin-embedded tumor tissues; (d) confirmed postoperative histopathology diagnosis. A total of 134 patients were excluded based on the following exclusion criteria: (a) incomplete clinical data; (b) incomplete follow-up information; (c) diagnosed as non-urothelial carcinoma; (d) diagnosed as non-muscle invasive bladder cancer; (e) with other malignancies; (f) received neoadjuvant chemotherapy or radiotherapy; (g) tissue detachment during immunohistochemistry (IHC) staining process. The detailed flowchart for patients' filtration of both cohorts is presented in Supplementary Figure 1. Finally, Zhongshan Cohort and FUSCC Cohort were comprised of 141 and 118 MIBC patients for analysis, respectively. Cisplatin-based combination adjuvant chemotherapy was applied for 119 patients of the 2 cohorts which lasted at one therapeutic cycle at least. The follow-up protocol was based on the European Association of Urology (EAU) guidelines for MIBC. The follow-up visits comprised medical history, physical examination and laboratory measurement every 3 months in the first year, every 6 months in the second year and once per year routinely afterward. The Cancer Genome Atlas (TCGA) data set was obtained from

<http://www.cbioportal.org> in Feb. 2018. In total, 406 MIBC patients were feasible for analysis with available clinical data and follow-up information.

### Assay methods

IHC staining was performed on formalin-fixed, paraffin-embedded tumor microarrays (TMA) as described previously.<sup>18</sup> To ensure an objective comparison of samples for evaluation, we performed IHC staining on an entire set of TMA each time. The IHC antibodies for molecules and immune cells staining are listed in Supplementary Table 1 and Supplementary Table 2. TMA slides of PDPN and PD-L1 IHC staining were scanned with Image-Pro Plus 6.0. TMA slides of other cells and molecules staining were scanned with NanoZoomer-XR (Hamamatsu). A PDPN<sup>+</sup> lymphatic vessel was defined as a cluster of endothelial cells or a single endothelial cell positive for PDPN staining.<sup>19</sup> Natural killer cells (NK cells) were quantified as the percentage of stained-positive cells in each section. Tumor-infiltrating PDPN<sup>+</sup> cell counts, PD-L1<sup>+</sup> immune cell counts, other stained-positive cell counts and intratumoral PDPN<sup>+</sup> lymphatic vessel counts were calculated as the mean value of 3 randomized high power magnification fields (HPF,  $\times 200$  magnification) of each section by Dr. Chen and Dr. Zhang who were blind to follow-up and clinical data. A semi-quantitative approach was applied to assign the IHC score of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression. In brief, the staining intensity was graded as 0 (negative staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining), while the proportion was scored as the percentage of positive cells (0 - 100%). The IHC score ranging from 0 to 300 was generated with the multiplication of staining intensity and proportion. The representative images of molecules and immune cells are illustrated in Supplementary Figure 2 and Supplementary Figure 3, and the representative image of intratumoral PDPN<sup>+</sup> lymphatic vessels is illustrated in Supplementary Figure 4A. The cutoff value for classifying tumor-infiltrating PDPN<sup>+</sup> cells high/low groups was 18 cells/HPF, which was determined by the median value of tumor-infiltrating PDPN<sup>+</sup> cells in Zhongshan Cohort. This cutoff value was then applied in FUSCC Cohort for consistency.

### Statistical methods

Overall survival (OS) was calculated from the date of operation until the date of death or last follow-up. Patients alive at last follow-up were censored for OS. Recurrence-free survival (RFS) was calculated from the date of operation until the date of first recurrence or last follow-up. Patients alive and without recurrence were censored for RFS.

ICK score was defined as the mean expression value of PD1, PD-L1, CTLA-4, LAG-3 and TIM-3. Effector score was defined as the mean expression value of granzyme B (GZMB), interferon- $\gamma$  (IFN- $\gamma$ ) and perforin (PRF1) while TNF- $\alpha$  was excluded because of its semi-quantitative evaluation approach. The cutoff value for PDPN mRNA expression in TCGA was determined by the median value. The gene expression signature scores for luminal signature, basal signature, p53-like signature and cell cycle signature were

obtained from the previous study.<sup>10</sup> The gene expression scores for FGFR3 activation signature,<sup>20</sup> T-cell-inflamed signature,<sup>21</sup> EGFR activation signature (gene members of BIOCARTA\_EGF\_PATHWAY from gene set enrichment analysis)<sup>22</sup> and proliferation signature<sup>23</sup> were defined by gene set variation analysis.<sup>24</sup> Gene set signature list is presented in Supplementary Table 3.

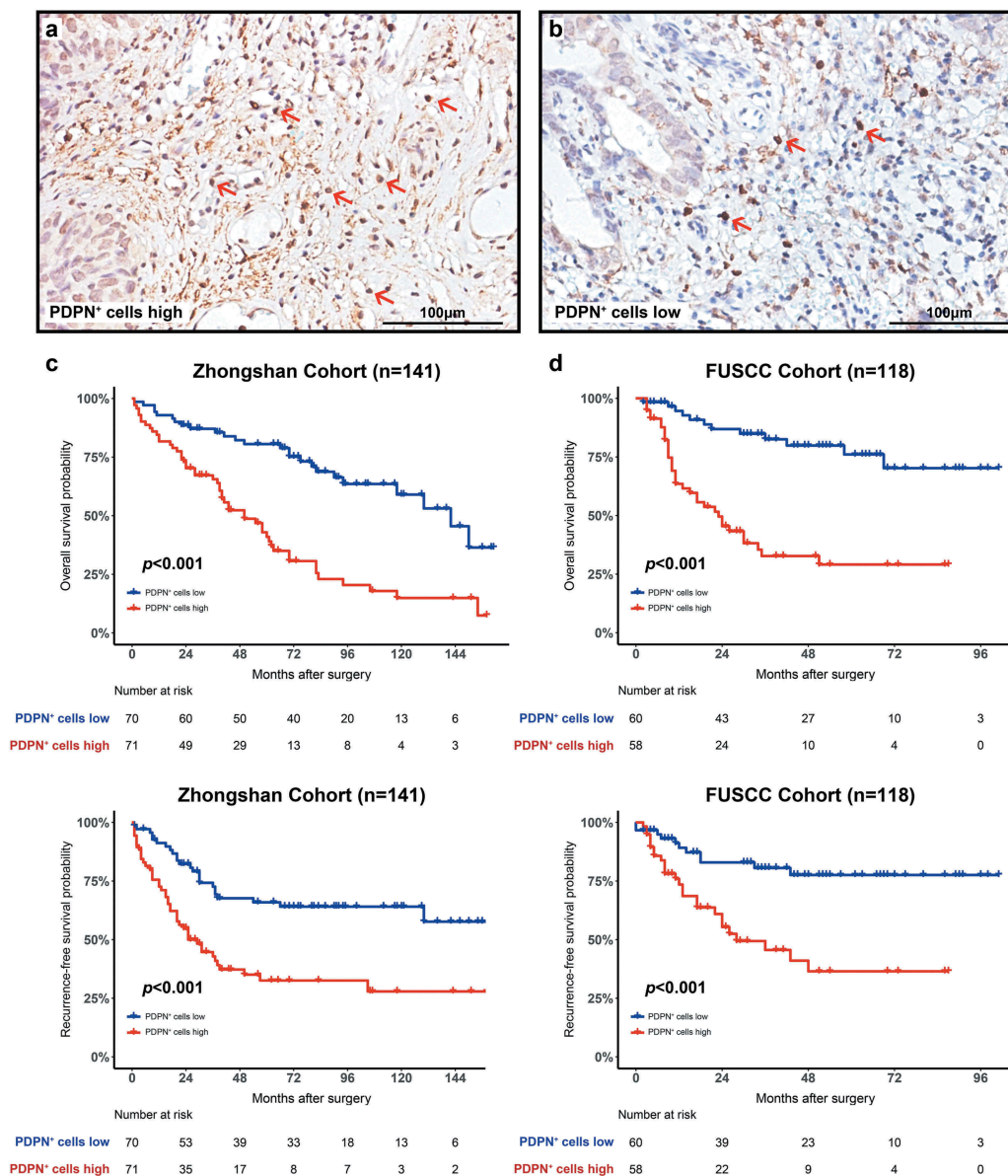
SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was utilized for statistical analyses. Student t-test and Chi-square test were conducted in patient characteristics analysis. Mann-Whitney U test, Spearman correlation analysis and Kruskal-Wallis H test were applied for statistical *p* values in the analyses of immune infiltration and molecular characterization. Survival outcomes were analyzed by Kaplan-Meier curves, log-rank test and univariate/multivariate Cox regression analysis. *P* values less than 0.05 were considered

statistically significant. Data were shown as mean  $\pm$  SD in this study.

## Results

### Tumor-infiltrating PDPN<sup>+</sup> cell abundance indicates inferior survival outcomes

Tumor-infiltrating PDPN<sup>+</sup> cells were quantified in MIBC (representative images are presented in Figure 1a,b). Patient characteristics of both cohorts are listed in Table 1. PDPN<sup>+</sup> cell infiltration of both cohorts significantly correlated with clinicopathological features (pT stage, pN stage and AJCC stage). Meanwhile, intratumoral PDPN<sup>+</sup> lymphatic vessels were evaluated and the positive rate was 55.2% in 259 MIBC patients (representative images are presented in Supplementary Figure



**Figure 1.** Prognostic significance of tumor-infiltrating PDPN<sup>+</sup> cell abundance. Representative images of PDPN immunohistochemistry staining showing tumor-infiltrating PDPN<sup>+</sup> cells high (a) and low (b) infiltration in MIBC. Kaplan-Meier curves for OS with PDPN<sup>+</sup> cell infiltration strata in Zhongshan Cohort (c) and FUSCC Cohort (d). Kaplan-Meier curves for RFS with PDPN<sup>+</sup> cell infiltration strata in Zhongshan Cohort (e) and FUSCC Cohort (f). Log-rank *p* values were shown.

**Table 1.** Patient characteristics of Zhongshan cohort and FUSCC cohort.

Parameters	Zhongshan Cohort (n = 141)			FUSCC Cohort (n = 118)		
	PDPN <sup>+</sup> cells low (n = 70)	PDPN <sup>+</sup> cells high (n = 71)	<i>p</i> value	PDPN <sup>+</sup> cells low (n = 60)	PDPN <sup>+</sup> cells high (n = 58)	<i>p</i> value
Age, years			0.331			0.403
Median (IQR)	61 (54–71)	64 (58–72)		61 (56–66)	63 (57–70)	
Size, cm			0.540			0.100
Median (IQR)	3.8 (2.4–4.6)	3.5 (2.5–5.0)		3.5 (2.7–5.0)	3.8 (2.8–4.0)	
Gender			0.682			0.642
Male	59	58		51	51	
Female	11	13		9	7	
Grade			0.067			0.077
Low grade	16	8		9	3	
High grade	54	63		51	55	
pT stage			0.001			0.067
pT2	55	35		31	18	
pT3	11	19		21	31	
pT4	4	17		8	9	
pN stage			0.030			0.007
pN0	69	64		48	33	
pN+	1	7		12	25	
AJCC stage			<0.001			0.006
II	55	32		20	14	
III	14	32		18	19	
IV	1	7		12	25	
LVI			0.004			0.190
Absent	34	18		44	36	
Present	36	53		16	22	
ACT			0.014			0.043
Applied	27	42		40	28	
Not applied	43	29		20	30	
Events						
Death	25	50		12	35	
Recurrence	24	44		11	26	

Abbreviations: IQR: interquartile range; AJCC: American Joint Committee on Cancer; LVI: lymphatic vessel invasion; ACT: adjuvant chemotherapy.

*p* value from Fisher's exact test was used when data failed to meet the requirement of Chi-square test.

*p* value < 0.05 marked in bold font shows statistically significant.

Characteristic age and size were analyzed by Student t-test.

4a comparing with tumor-infiltrating PDPN<sup>+</sup> cells). There is no correlation between tumor-infiltrating PDPN<sup>+</sup> cells and intratumoral PDPN<sup>+</sup> lymphatic vessels (Supplementary Figure 4B). The clinical significance of tumor-infiltrating PDPN<sup>+</sup> cells was next explored. Median follow-up was 74.5 months (Zhongshan Cohort) and 44.5 months (FUSCC Cohort). Kaplan-Meier analysis and log-rank test were applied for comparing OS and RFS. PDPN<sup>+</sup> cells high infiltration was associated with significantly inferior OS in both cohorts ( $p < .001$  and  $p < .001$ ; Figure 1c,d). The 5-y OS was estimated at 41.0% and 28.9% in patients with PDPN<sup>+</sup> cell high infiltration compared with 80.6% and 76.0% in patients with PDPN<sup>+</sup> cell low infiltration in both cohorts, respectively. Patients with PDPN<sup>+</sup> cell high infiltration also had worse RFS in both cohorts ( $p < .001$  and  $p < .001$ ; Figure 1e,f). The 5-y RFS was estimated at 32.5% and 36.3% in patients with PDPN<sup>+</sup> cell high infiltration compared with 65.9% and 77.6% in patients with PDPN<sup>+</sup> cell low infiltration in both cohorts, respectively. We found that PDPN<sup>+</sup> cell infiltration was an independent adverse prognostic factor for OS and RFS according to univariate (Supplementary Table 4) and multivariate (Supplementary Table 5) Cox regression analyses.

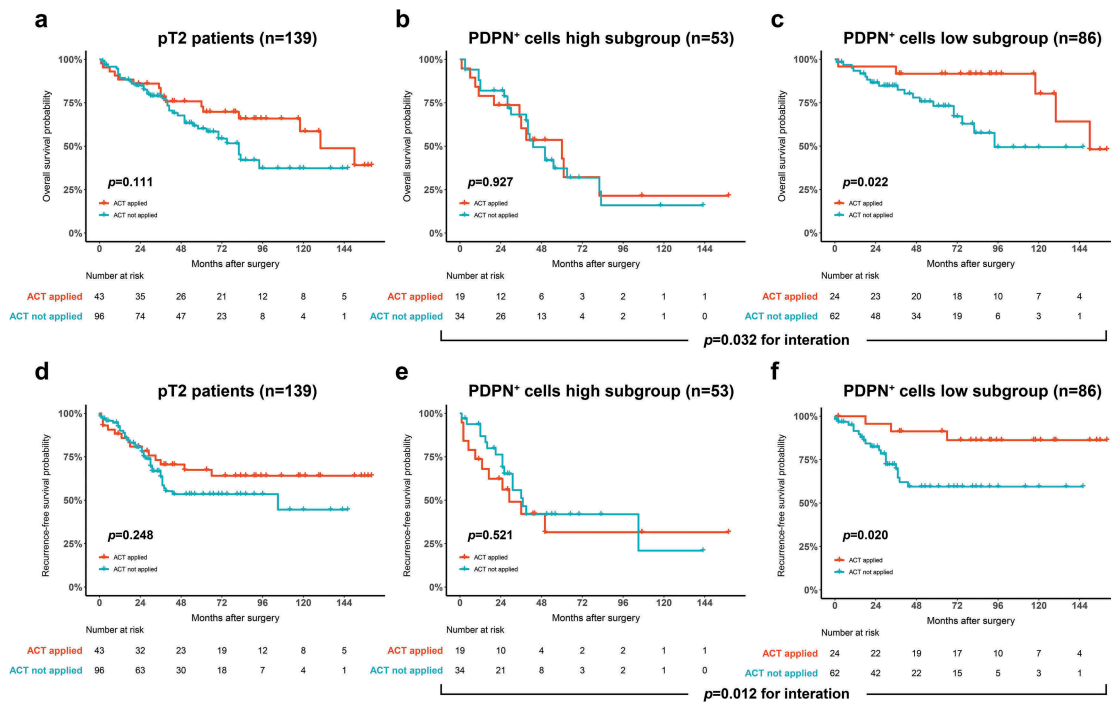
### Tumor-infiltrating PDPN<sup>+</sup> cell abundance yields poor adjuvant chemotherapeutic response

The association between PDPN<sup>+</sup> cell infiltration and therapeutic responsiveness of cisplatin-based ACT was further investigated. We combined the two cohorts for exploration. ACT application did not improve OS of pT2 MIBC patients

( $p = .111$ ; Figure 2a) or PDPN<sup>+</sup> cells high subgroup ( $p = .927$ ; Figure 2b). However, ACT application led to a superior OS in PDPN<sup>+</sup> cells low subgroup ( $p = .022$ ; Figure 2c). The RFS showed no improvement with ACT application in pT2 MIBC patients ( $p = .248$ ; Figure 2d) or PDPN<sup>+</sup> cells high subgroup ( $p = .521$ ; Figure 2e). Nevertheless, there was an improved RFS in PDPN<sup>+</sup> cells low subgroup with ACT application ( $p = .020$ ; Figure 2f). Univariate cox regression analysis was performed to assess the relationship between PDPN<sup>+</sup> cell infiltration and ACT benefit, which suggested that pT2 MIBC patients with PDPN<sup>+</sup> cell low infiltration could receive more benefit from adjuvant chemotherapy (OS: HR, 0.290, 95% CI, 0.095 ~ 0.884,  $p = .030$ , RFS: HR, 0.262, 95% CI, 0.077 ~ 0.887,  $p = .031$ ) while no ACT benefit was observed in patients with pT3 + 4 MIBC or its subgroups according to PDPN<sup>+</sup> cell stratification (Supplementary Table 6).

### Tumor-infiltrating PDPN<sup>+</sup> cell abundance correlates with immunoevasive contexture

We investigated whether tumor-infiltrating PDPN<sup>+</sup> cells were associated with the tumor immune microenvironment which closely related to prognosis, adjuvant treatment responsiveness and other parameters.<sup>25</sup> Through evaluating PDPN<sup>+</sup> cell infiltration with the effector score and effector molecules (IFN- $\gamma$ , GZMB, PRF1 and TNF- $\alpha$ ; Figure 3a), we found that the effector score decreased significantly in PDPN<sup>+</sup> cell high infiltration group ( $p = .036$ ; Figure 3b) indicating an impaired anti-tumor function. PDPN<sup>+</sup> cell infiltration adversely correlated with



**Figure 2.** Predictive value of tumor-infiltrating PDPN<sup>+</sup> cell abundance for suboptimal adjuvant cisplatin-based chemotherapy responsiveness. Kaplan–Meier curves for OS comparing ACT applied versus not applied in all pT2 patients (a), PDPN<sup>+</sup> cells high subgroup (b) and PDPN<sup>+</sup> cells low subgroup (c). Kaplan–Meier curves for RFS comparing ACT applied versus not applied in all pT2 patients (d), PDPN<sup>+</sup> cells high subgroup (e) and PDPN<sup>+</sup> cells low subgroup (f). Log-rank  $p$  values were shown.

GZMB and TNF- $\alpha$  expression (Spearman's R:  $-0.190$  and  $-0.236$ ,  $p = .024$  and  $p = .005$ ; Figure 3c). The association between PDPN<sup>+</sup> cell infiltration and other ICK molecules (PD-1, PD-L1, CTLA-4, TIM-3 and LAG-3) was next evaluated (Figure 3d). The ICK score was higher in PDPN<sup>+</sup> cell high infiltration group than PDPN<sup>+</sup> cell low infiltration group ( $p = .005$ ; Figure 3e). PDPN<sup>+</sup> cell infiltration strongly correlated with PD-1 and PD-L1 expression (Spearman's R:  $0.288$  and  $0.351$ ,  $p = .001$  and  $p < .001$ ; Figure 3f) and was also associated with LAG-3 and TIM-3 expression (Spearman's R:  $0.241$  and  $0.255$ ,  $p = .004$  and  $p = .002$ , Supplementary Figure 5B). Previously, we identified an immune cell infiltration classification (immunotype A/B) based on the infiltration level of CD8<sup>+</sup> T cells, NK cells, regulatory T cells (Tregs), macrophages and mast cells, which could be applied for predicting survival outcome and ACT benefit.<sup>26</sup> We sought to find the association between PDPN<sup>+</sup> cell infiltration and immune contexture (Figure 3g). PDPN<sup>+</sup> cells infiltrated more in immunotype B tumors compared with immunotype A tumors ( $p = .005$ ; Figure 3h). Moreover, increased PDPN<sup>+</sup> cell infiltration was significantly associated with more Tregs and M2 macrophage infiltration (Spearman's R:  $0.227$  and  $0.192$ ,  $p = .007$  and  $p = .023$ ; Figure 3i). The comprehensive correlation analyses between PDPN<sup>+</sup> cells and molecules or immune cells were demonstrated in Supplementary Figure 5A-D.

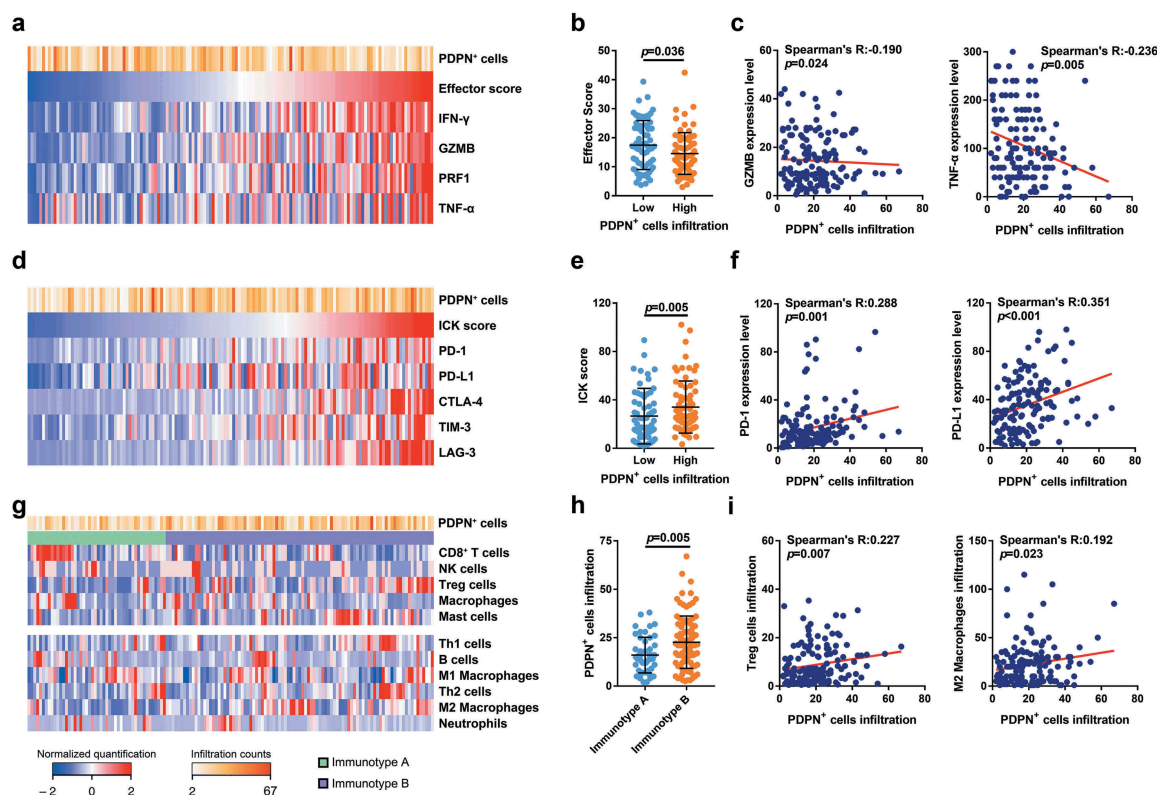
### Molecular correlates in intratumoral PDPN abundant TCGA cohort patients

We found that PDPN mRNA expression in TCGA showed difference across molecular subtypes. PDPN mRNA expression accumulated in luminal-infiltrated and basal-squamous

subtypes compared with luminal-papillary subtype by 2017 TCGA classification ( $p < .001$ ; Figure 4a).<sup>10</sup> Meanwhile, there was a higher PDPN mRNA expression in stroma-rich and Ba/Sq subtypes than LumP and LumU subtypes by the consensus classification ( $p < .001$ ; Figure 4b).<sup>11</sup> Next, we applied the molecular subtype signature to further investigate the association between PDPN mRNA expression and molecular characteristics. Tumors with high PDPN mRNA expression showed a low level of luminal signature ( $p < .001$ ) and a high level of basal signature and p53-like signature ( $p < .001$  and  $p < .001$ ; Figure 4c). Previous studies illustrated that molecular classification may stratify patients for personalized anti-tumor therapies.<sup>4</sup> FGFR3 activation signature decreased ( $p < .001$ ) while T-cell-inflamed signature and EGFR activation signature elevated in tumors with high PDPN mRNA expression ( $p < .001$  and  $p < .001$ ; Figure 4d). However, PDPN mRNA expression showed no correlation with cell cycle signature and proliferation signature ( $p = .368$  and  $p = .292$ ; Figure 4e).

## Discussion

The low response rate of ACT for MIBC patients remains an intractable clinical issue.<sup>3</sup> Stratification of patients who are resistant or sensitive to first-line chemotherapy has a direct clinical significance for proper therapies. It was previously unveiled that bladder tumors with high MSH2 protein<sup>27</sup> or somatic ERCC2 mutation<sup>28,29</sup> received superior response from platinum-based chemotherapy. There is an expanding focus on the involvement of immune system in the cytotoxic chemotherapy process.<sup>30</sup> Our previous studies concentrated on the contribution of immune contexture to the beneficial of ACT in MIBC including tumor-infiltrating mast cells,<sup>31</sup> neutrophils,<sup>32</sup> B cells<sup>33</sup> and an



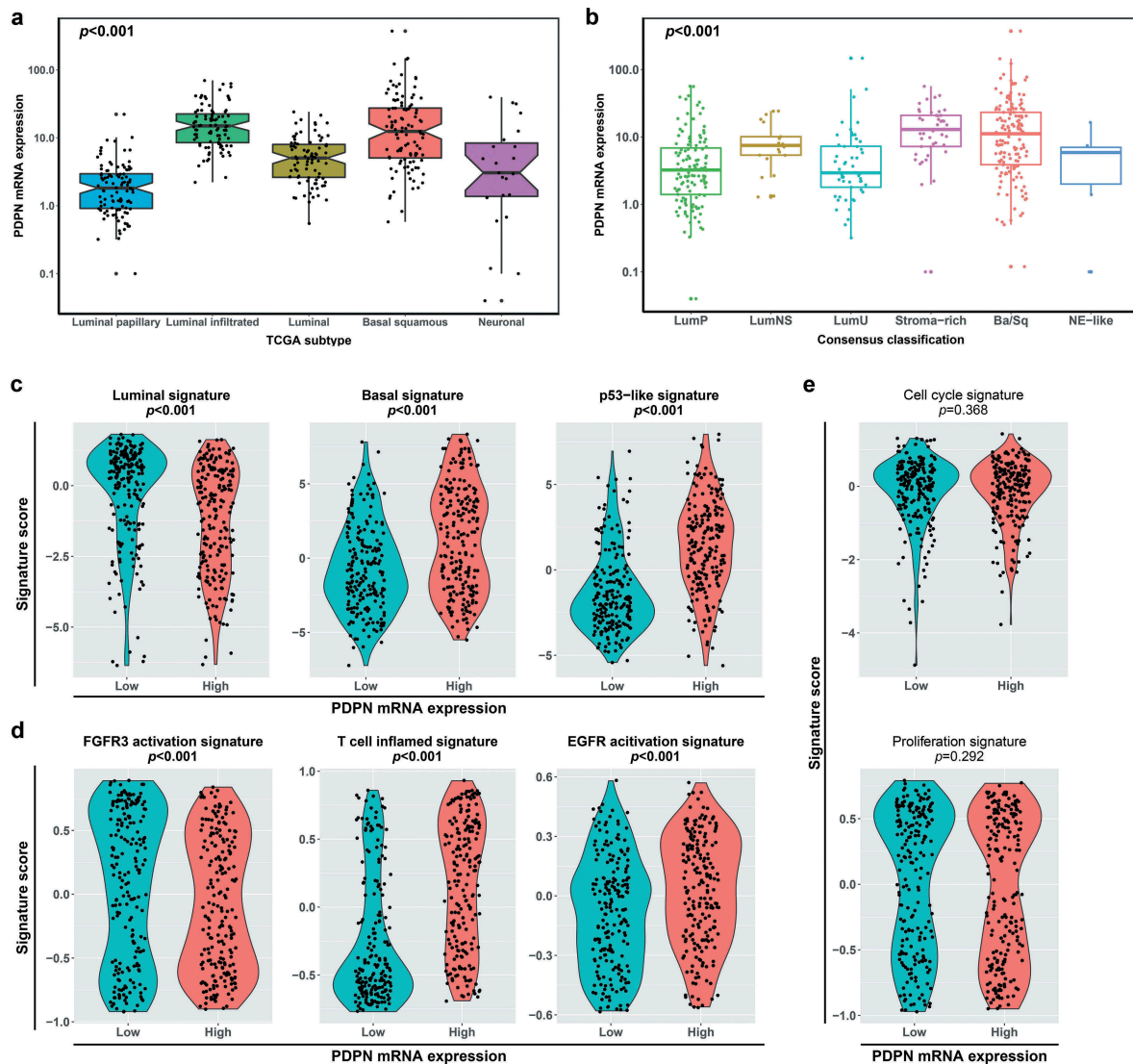
**Figure 3.** Characterization of the immunoevasive microenvironment with tumor-infiltrating PDPN<sup>+</sup> cell abundance. (a) Heatmap showing PDPN<sup>+</sup> cell infiltration and effector molecules (IFN- $\gamma$ , GZMB, PRF1 and TNF- $\alpha$ ) expression in Zhongshan Cohort (n = 141). (b) Evaluation of effector score in PDPN<sup>+</sup> cells high/low infiltration groups (n = 141). (c) Correlation between PDPN<sup>+</sup> cell infiltration and GZMB or TNF- $\alpha$  expression (n = 141). (d) Heatmap showing PDPN<sup>+</sup> cell infiltration and immune checkpoint (ICK) molecules (PD-1, PD-L1, CTLA-4, TIM-3 and LAG-3) expression in Zhongshan Cohort (n = 141). (e) Evaluation of ICK score in PDPN<sup>+</sup> cells high/low infiltration groups (n = 141). (f) Correlation between PDPN<sup>+</sup> cell infiltration and PD-1 or PD-L1 expression (n = 141). (g) Heatmap showing PDPN<sup>+</sup> cell infiltration, immunotype A/B distribution, immune cells involved in immunotype A/B definition (CD8<sup>+</sup> T cells, NK cells, Tregs, macrophages and mast cells) and other anti-tumor or pro-tumor immune cells (Th1 cells, B cells, M1 macrophages, Th2 cells, M2 macrophages and neutrophils) infiltration (n = 141). (h) Distribution of PDPN<sup>+</sup> cell infiltration in immunotype A/B groups (n = 141). (i) Correlation between PDPN<sup>+</sup> cell infiltration and Tregs or M2 macrophage infiltration (n = 141). Data were analyzed by Mann–Whitney U test and Spearman’s rank correlation test.

immunotype A/B classification.<sup>26</sup> As a brand-new ICK,<sup>17</sup> PDPN was first identified as a prognosticator and a predictive biomarker for adjuvant cisplatin-based chemotherapy responsiveness in MIBC patients. The intrinsic relationship between tumor-infiltrating PDPN<sup>+</sup> cells and ACT resistance is worth investigating. Moreover, targeting PDPN synergizes with adjuvant chemotherapy may be considered in the future.

Originally, PDPN is applied as a lymphatic vessel marker in multiple cancer types including bladder cancer.<sup>34</sup> Beyond its ability to mark lymphatic endothelial cells, we identified a poor prognosis and immunoevasive subtype MIBC with tumor-infiltrating PDPN<sup>+</sup> cell abundance. The study of PDPN being a T cell ICK elucidates that PDPN deficiency in CD8<sup>+</sup> T cells results in an enhancement of TNF- $\alpha$  production,<sup>17</sup> which is partly consistent with our finding that PDPN<sup>+</sup> cell infiltration correlated with diminished TNF- $\alpha$  expression in tumor microenvironment. Furthermore, we revealed that PDPN<sup>+</sup> cell infiltration correlated with other ICK expression including PD-1, PD-L1, LAG-3 and TIM-3. Previous studies demonstrated that the frequency of Foxp3<sup>+</sup> Tregs was significantly elevated in mice with T-cell-specific overexpression of PDPN compared with the wild type.<sup>35</sup> It was also reported that PDPN-deficient macrophages impaired the expression of *Arg1*, a phenotype marker for M2 macrophages.<sup>15</sup> Through comparing the immune contexture

between PDPN<sup>+</sup> cells high and low infiltration groups, we found that PDPN<sup>+</sup> cell abundance identified the immune contexture with increased Tregs and M2 macrophage infiltration in MIBC which were both associated with immunosuppression leading to poor prognosis.<sup>25,30</sup>

There is a growing recognition that molecular classification may predict tumor progression and provide information on effective therapeutic responsiveness, including ACT, ICK blockade therapies and targeted therapies.<sup>4,5</sup> We found that tumors with high PDPN expression could present a basal/squamous-like characterization, which was associated with poor prognosis, high EGFR and its ligand expression and high ICK expression.<sup>10,11</sup> High T-cell-inflamed signature was also observed in high PDPN expression group, which was a marker for favorable response to anti-PD-L1 immunotherapy.<sup>21</sup> Thus, we inferred that PD-L1 blockade immunotherapy or EGFR-targeted therapy may potentially take effect in these tumors. We also revealed that PDPN expression correlated with p53-like signature, which is enriched in luminal-infiltrated and stroma-rich subtypes<sup>10,11</sup> and previously reported as a marker for predicting resistance to neoadjuvant chemotherapy.<sup>36</sup> Meanwhile, low PDPN expression was detected in tumors with luminal-papillary or LumP subtype featuring with good prognosis and FGFR3 mutation.<sup>10,11</sup> FGFR3-targeted therapy may be an encouraging choice for low



**Figure 4.** Characterization of PDPN mRNA expression across molecular subtypes. Comparison of the PDPN mRNA expression across 2017 TCGA classification (a) and consensus classification (b) in TCGA cohort ( $n = 406$ ). (c) Comparison of the expression of molecular subtype signatures (luminal signature, basal signature and p53-like signature) between high and low PDPN mRNA expression groups. (d) Comparison of the expression of therapy-associated signatures (FGFR3 activation signature, T-cell-inflamed signature and EGFR activation signature) between high and low PDPN mRNA expression groups. (e) Comparison of the expression of cell cycle signature and proliferation signature between high and low PDPN mRNA expression groups. Data were analyzed by Kruskal–Wallis H test (a, b) and Mann–Whitney U test (c–e).

PDPN expression tumors. Reclassification of bladder cancer by molecular heterogeneity provided an innovative approach to improving the therapeutic effectiveness. PDPN expression possessed the potential to classify MIBC subgroups and guide personalized anti-tumor therapies. The therapy-related predictive value of PDPN and the detailed mechanisms between PDPN and certain therapies need more investigation through in vitro or in vivo experiments.

In summary, tumor-infiltrating PDPN<sup>+</sup> cells were identified as an independent prognosticator for survival outcome and a predictive biomarker for poor ACT responsiveness, highlighting its clinical significance in MIBC. Tumors with PDPN<sup>+</sup> cell abundance infiltrated intense immunosuppressive contexture. Intratumoral PDPN expression has a relationship with MIBC molecular classification and therapy-related signatures. PDPN could be a potential immunotherapeutic target for treating MIBC.

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## Consent for publication

All authors provide their consent for publication of the manuscript.

## Availability of data and materials

All data generated that are relevant to the results presented in this article are included in this article. Other data that were not relevant for the results presented here are available from the corresponding author Dr. Xu upon reasonable request.

## Author contributions

Q. Zhou, Z. Wang, H. Zeng and H. Zhang for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript; Z. Liu, Q. Huang, J. Wang, Y. Chang, Q. Bai, L. Liu, Y. Zhu, L. Xu, B. Dai and J. Guo for technical and material support; Y. Xia, Y. Wang and J. Xu for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding and study supervision. All authors read and approved the final manuscript.

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