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Programmed death ligand-1 is associated with tumor infiltrating lymphocytes and poorer survival in urothelial cell carcinoma of the bladder

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Drugs blocking programmed death ligand-1 (PD-L1) have shown unprecedented activity in metastatic and unresectable bladder cancer. The purpose of the present study was to investigate the expression, clinical significance and association of PD-L1 with tumor-infiltrating lymphocytes (TIL) in resectable urothelial cell carcinoma of the bladder (UCB). In this retrospective study, 248 UCB patients who received radical cystectomy or transurethral resection were examined. Immunohistochemistry was used to evaluate PD-L1 expression and stromal CD8^{+} TIL, Th1 orientation T cell (T-bet^{+}) and PD-1^{+} TIL densities within the intratumoral regions and associated stromal regions. Of the 248 specimens, 23% showed PD-L1 expression in tumor cells and 55% in tumor-infiltrating immune cells. CD8^{+} TIL, T-bet^{+} TIL and PD-1^{+} TIL were distributed throughout the tumor tissues and were more frequently distributed in stromal regions than in intratumoral regions. PD-L1^{+} tumor cells and PD-L1^{+} immune cells were positively associated with aggressive clinical features (all $P < .05$). Both PD-L1^{+} tumor cells and PD-L1^{+} immune cells were associated with poorer recurrence-free and overall survival (all $P < .05$). Multivariate analysis showed that PD-L1^{+} immune cells were an independent prognostic factor for overall ($P = .001$) and recurrence-free survival ($P = .024$). Notably, high stromal CD8^{+} TIL and PD-1^{+} TIL density were associated with poorer overall survival ($P = .031$ and $P = .001$, respectively). In the stroma, CD8^{+} TIL density has strong positive association with PD-L1^{+} immune cells and PD-1^{+} TIL density (all $P < .0001$). These results suggested that an exhausted immune state occurred in the tumor stroma in UCB. Further clinical development of immune-checkpoint inhibitors may be effective for resectable patients with UCB.

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1 | INTRODUCTION

The prognosis and treatment of UCB have improved little since the 1980s.[1] Comprehensive molecular characterizations in recent genome-wide expression and sequencing studies have indicated several subtypes of bladder cancer.[2,3] However, it is unclear whether improved tumor stratification based on these molecular classifications will provide clinical benefit by enhancing targeted therapy. Interestingly, TCGA indicates UCB contains more somatic mutations than most common cancers, and may contain high levels of tumor-specific neoantigens.[4] BCG therapy exerts an antitumor effect in bladder cancer by activation of the immune system, indicating immune status may represent a target for and marker of effective anti-cancer immunotherapies in UCB.[5]

Tumor progression is the product of crosstalk between different cell types within tumors.[6,7] High densities of T cells with a Th1 orientation (T-bet^{+}) and cytotoxic CD8^{+} TIL have been associated with good clinical outcome in many tumor types, including melanoma and breast, colorectal and lung cancer.[8] However, the prognostic value of TIL in UCB has been controversial.[9-11] Sharma et al[9] found that high intratumoral CD8^{+} TIL had better survival in advanced UCB. In contrast, Lipponen et al[10] observed that CD8^{+} TIL in the tumor stroma were correlated with poor OS. In addition, Horn et al[11] reported that the ratio of Treg among CD3^{+} or CD8^{+} TIL indicated a poor prognosis with invasive UCB. Our previous studies found intratumoral CD103^{+}CD8^{+} tissue-resident memory T cells were associated with a favorable prognosis in UCB. However, stromal CD8^{+} TIL density was inversely associated with clinical outcomes,[12,13] which indicates a role for tumor-promoting proinflammatory and T-cell dysfunction, underlining the importance of the spatial distribution of immune cells within the tumor landscape.

Programmed death-ligand 1, an immune checkpoint in the B7/CD28 pathway, negatively regulates T-lymphocyte migration, proliferation and function by binding to its receptors PD-1 or B7.1 (CD80).[14,15] PD-L1 is expressed by tumor cells and stromal cells, such as macrophages, neutrophils, T cells and B cells; PD-1 is mainly expressed during initial T-cell activation and remains upregulated on exhausted, dysfunctional TIL.[15] PD-L1 is regulated by many proinflammatory cytokines involved in cellular immune function, most potently by interferon-γ (IFN-γ) secreted by effector T cells.[16] Notably, anti-PD1/PD-L1 therapy has become the backbone of immunotherapy for several tumor types, including metastatic bladder cancer, melanoma and lung cancer.[17-19] In last 2 years, five checkpoint blockade therapies, targeting the PD-1/PD-L1 axis, have been approved for metastatic and unresectable UCB treatment.[20] However, the associations between PD-L1 expression and prognosis vary in different malignancies.[21] In UCB, it has been found that patients with higher PD-L1-positive tumor cells experienced higher frequencies of recurrence and poorer survival following cystectomy.[22-24] In contrast, a recent study found that PD-L1-positive tumor cells were not predictive of OS, but positive PD-L1 expression in stromal cells was significantly associated with longer survival in patients who developed metastases and received subsequent chemotherapy.[25] Therefore, the role of PD-L1 expression in resectable UCB as a predictive biomarker remains less unclear.

In the present study, we sought to evaluate PD-L1 expression in all stages of resectable UCB, including the INT and ST. We also assessed whether PD-L1/PD-1 expression, stromal CD8^{+} TIL density and Th1 orientation T cell (T-bet^{+}) density are associated with survival outcomes.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue specimens

A total of 248 patients with pathologically confirmed UCB treated at Sun Yat-sen Memorial Hospital between June 2006 and December 2012 were retrospectively assessed. All patients underwent cystectomy or transurethral resection for UCB; no patients received preoperative therapy. Tumors were graded according to the World Health Organization 2004 classification and staged according to the TNM classification (8th edition, 2016). Clinicopathological characteristics of the patients are summarized in Table S1. Written informed consent was obtained from all patients; the protocol was approved by the review board of the hospital.

Postoperative follow up was conducted at 3-monthly intervals in the first year, 6-monthly intervals during the second year, and annually thereafter.[12,26] Median follow up for surviving patients was 52 months (range, 16-104 months). OS was defined as the interval between surgery and death or last observation, RFS, to recurrence or last observation. Of the 248 patients, 63 (25%) died, 69 (28%) had tumor recurrence, and 161 (65%) survived without recurrence during follow up.

2.2 | Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm) were processed for immunohistochemistry as previously described,[9,10] using antibodies against human PD-L1 (1:100; Spring Bioscience, Pleasanton, CA, USA), CD8 (1:200; Thermo Fisher Scientific, Waltham, MA, USA), T-bet (1:200; Cell Signaling Technology, Danvers, MA, USA),

PD-1 (1:200; Cell Signaling Technology) or control antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and secondary antimouse/rabbit HRP-conjugated antibodies (Envision+ Dual Link Kit; Dako, Glostrup, Denmark). Sections were developed using 3,3′-diaminobenzidine tetrahydrochloride with the Envision System (Dako).

2.3 | Evaluation of immunohistochemical staining

Tissue sections were analyzed by two independent pathologists blinded to clinicopathological and survival data. PD-L1 expression was evaluated on TC and IC as reported in recent trials.[17,27] An isotype control was used as negative control for signal detection and both TC and IC typically showed clear positive membranous staining with a variably strong component of cytoplasmic staining (Figure 1). For TC, proportion of PD-L1$^+$ TC was estimated as the percentage of total TC; for IC, the percentage of PD-L1$^+$ IC occupying the tumor was estimated. Specimens were scored as 0, 1, 2, or 3 if <1%, <5%, <10%, or ≥10% of cells per area were PD-L1$^+$, respectively (Figure S1). Density of CD8$^+$ TIL, T-bet$^+$ TIL and PD-1$^+$ TIL in the INT and ST regions was screened at low power (×100 magnification), and five representative high-power fields were assessed for every specimen (×400 magnification; 0.07 mm^2 per field). CD8$^+$ TIL, T-bet$^+$ TIL and PD-1$^+$ TIL were counted manually. Data are expressed as mean ± SE number of cells per field.

2.4 | Statistical analysis

Statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were determined using the Wilcoxon signed-rank test. Cumulative survival time was calculated using the Kaplan-Meier method and compared using the log-rank test. A multivariate Cox proportional hazards model was used to estimate adjusted hazard ratios and 95% CI and identify independent prognostic factors. PD-L1-positivity was defined as ≥5% PD-L1$^+$ TC or IC. For categorical analyses, median value was used as a cut-off to dichotomize continuous variables (for clinical applications). Two-tailed P-values <.05 were considered significant.

3 | RESULTS

3.1 | Programmed death ligand-1 expression in resectable UCB

Programmed death ligand-1 expression was clearly observed in the cell membranes of the 248 resectable UCB specimens, with variable cytoplasmic staining in TC and/or IC (Figure 1). PD-L1 IHC score distribution was 0 (183, 74%), 1 (7, 3%), 2 (15, 6%) and 3 (43, 17%) for TC and 0 (90, 36%), 1 (22, 9%), 2 (48, 19%) and 3 (88, 36%) for IC. Overall, 23% (IHC 2/3, 58/248) and 55% (IHC 2/3, 136/248) of specimens contained PD-L1$^+$ TC and PD-L1$^+$ IC, respectively. Moreover, 43 patients had PD-L1 IHC scores of 2 or 3 in both TC and IC.

3.2 | Programmed death ligand-1$^+$ IC and PD-L1$^+$ TC are associated with advanced clinical features

Programmed death ligand-1$^+$ TC and PD-L1$^+$ IC were positively associated with tumor size (P < .05), tumor stage (P < .001), nodal status (P < .05) and histological grade (P < .001). Moreover, PD-L1$^+$ IC was associated with age (P < .05) and PD-L1$^+$ TC was associated with metastasis (P < .05; Table 1). These results indicate that high PD-L1 expression is associated with aggressive clinical features in resectable UCB.

3.3 | Programmed death ligand-1$^+$ IC and PD-L1$^+$ TC are associated with poorer survival

In univariate analysis, tumor size, tumor stage, nodal status, metastatic status and histological grade were inversely associated with OS and RFS. For further analysis, we divided the 248 patients into two groups based on PD-L1$^+$ TC and/or PD-L1$^+$ IC. Kaplan-Meier analysis showed an inverse association between PD-L1 expression and OS (P = .002 for TC and P < .001 for IC; Figure 2A,C) and RFS (P < .001 for TC and P = .0006 for IC; Figure 2B,D). Both PD-L1$^+$ TC and PD-L1$^+$ IC were associated with significantly shorter OS (median, 38 vs 44 months for TC and 37 vs 52 months for IC) and RFS (median, 29 vs 42 months for TC and 31 vs 48 months for IC vs patients with PD-L1$^-$ TC or PD-L1$^-$ IC). Significant variables in univariate analysis
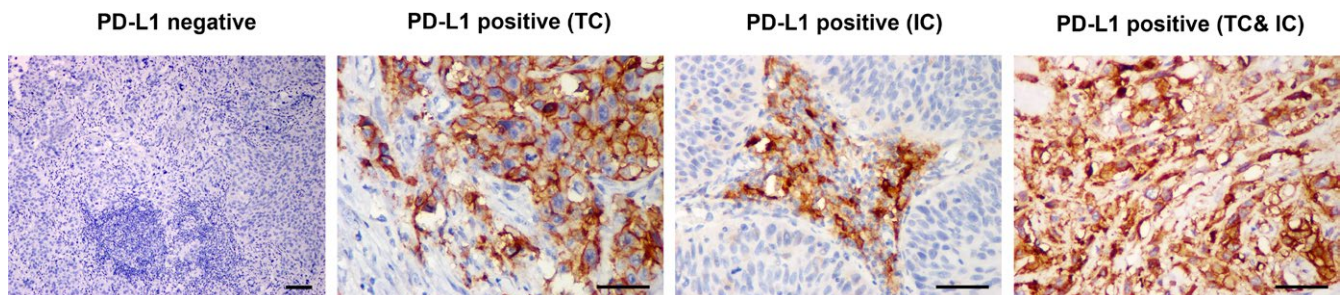
FIGURE 1 Expression of programmed death-ligand 1 (PD-L1) in human urothelial cell carcinoma of the bladder (UCB) (n = 248). Representative images of immunohistochemical detection of PD-L1 (brown) in tumor cells (TC), tumor-infiltrating immune cells (IC) or both TC and IC. PD-L1 positivity was defined as ≥5% PD-L1$^+$ TC or IC. PD-L1 negative image is 10× magnification (scale bar 100 μm); other images are 40× magnification (scale bar 50 μm)

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TABLE 1 Association of PD-L1 expression in tumor cells and tumor-infiltrating immune cells with clinical features of urothelial cell carcinoma of the bladder (n = 248)

Variable	PD-L1+ TC				PD-L1+ IC			
	Low	High	R	P	Low	High	R	P
No. of patients	190	58			112	136		
Age, years			0.07	0.271			0.138	**0.029**
≤60	81	20			54	47		
>60	109	38			58	89		
Gender			−0.054	0.397			−0.062	0.328
Male	162	52			94	120		
Female	28	6			18	16		
Tumor size, cm			0.128	**0.043**			0.142	**0.026**
≤3	77	15			50	42		
>3	113	43			62	94		
Multifocality			−0.1	0.117			−0.114	0.055
Unifocal	89	34			95	107		
Multifocal	101	24			50	34		
Tumor stage			0.289	**3.6×10^{-6}**			0.223	**4.1×10^{-4}**
Ta-T1	114	15			72	57		
T2-T4	76	43			40	79		
Nodal status			0.135	**0.033**			0.161	**0.011**
N0	166	44			102	108		
N1-N2	24	14			10	28		
Metastasis status	185	53	0.129	**0.043**	107	131	−0.02	0.755
M0	5	5			5	5		
M1								
Histological grade			0.227	**3×10^{-4}**			0.276	**1×10^{-5}**
Low	66	6			48	24		
High	124	52			64	112		

IC, tumor-infiltrating immune cells; PD-L1, programmed death ligand-1; TC, tumor cells; UCB, urothelial cell carcinoma of the bladder.
Differences between groups were calculated using the Wilcoxon signed-rank test. Significant P-values are shown in bold.

were adopted as covariates in multivariate analysis (Table 2), which showed that PD-L1+ IC was an independent prognostic factor for OS (HR: 3.02, P = .001) and RFS (HR: 1.858, P = .024).

We further evaluated the prognostic value of PD-L1 expression in subgroups of patients stratified by tumor stage (Figure S2). PD-L1+ IC maintained prognostic value for shorter OS and RFS, except for RFS in non-muscle-invasive bladder cancer (NMIBC, tumor stage Ta+T1, P = .24).

We also evaluated the combined influence of PD-L1+ TC plus PD-L1+ IC on patient outcomes using the Wald test for interaction ($P_{interaction}$). Patients were divided into three groups: (I) PD-L1− TC and PD-L1− IC (n = 97); (II) PD-L1+ TC or PD-L1+ IC (n = 108); and (III) PD-L1+ TC and PD-L1+ IC (n = 43). Significant differences in mortality and recurrence were found between groups I and III, as well as between groups I and II (Figure 2E,F). Median OS and RFS were 53 and 50 months for group I, 39 and 36 months for group II, and 34 and 26 months for group III, respectively. Multivariate analysis

confirmed that the differences in OS and RFS between groups I and III were significant (P = .007 and P = .002, respectively; Table S2). These data clearly show a strong association between PD-L1 expression and poorer survival in resectable UCB.

3.4 | Associations between PD-L1+ IC and PD-L1+ TC, CD8+ TIL, T-bet+ TIL and clinical outcomes

Programmed death 1 plays a dominant role in suppression of effector T-cell responses in vivo, especially in the tumor microenvironment.[15,16] To investigate the association between PD-L1 status and TIL, CD8+ TIL and Th1 originated T-bet expression (T-bet+ TIL) were evaluated in both the INT and ST regions of the UCB specimens. CD8+ TIL were distributed throughout the tumor tissues and were more frequent in ST than in INT (65 ± 4 and 18 ± 2 cells/field, respectively; Figure 3A,C; n = 128). T-bet+ TIL were also predominantly distributed in ST than in INT (54 ± 8 and 22 ± 5 cells/
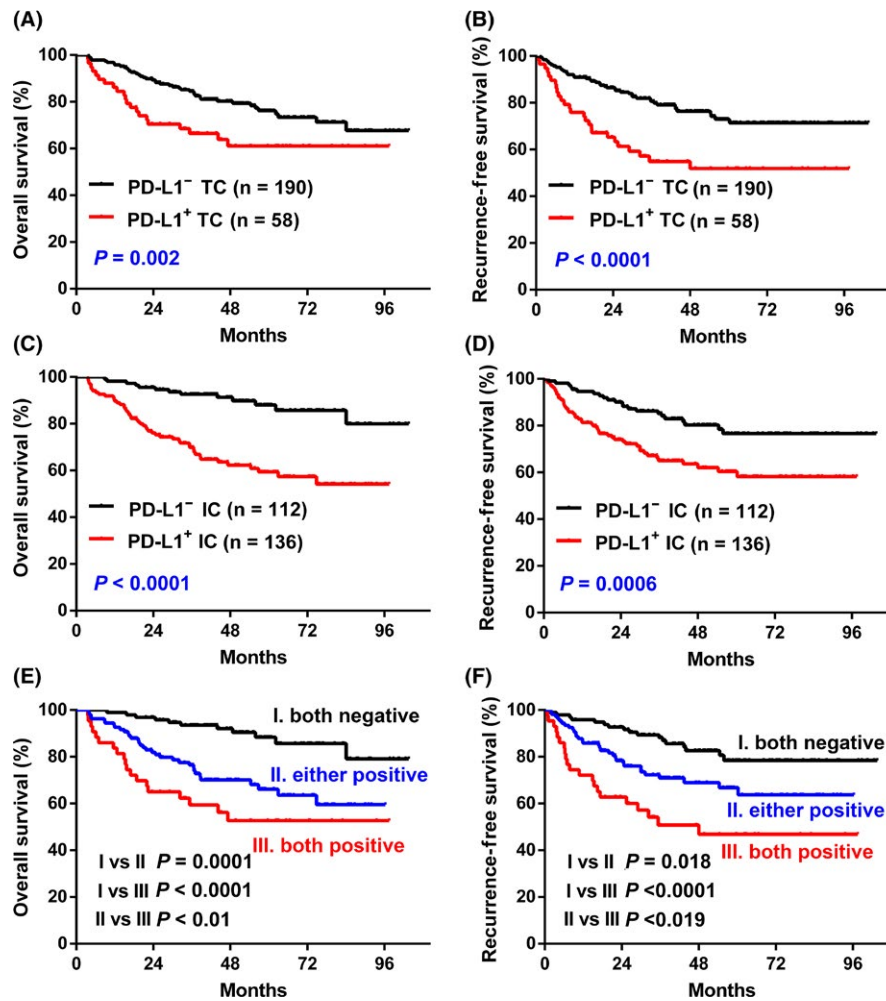
FIGURE 2 Kaplan-Meier overall and recurrence-free survival curves for patients with urothelial cell carcinoma of the bladder (UCB) stratified by (A,B) programmed death-ligand 1 (PD-L1)$^+$ tumor cells (TC), (C,D) PD-L1$^+$ tumor-infiltrating immune cells (IC) and (E,F) both PD-L1$^+$ TC and PD-L1$^+$ IC. *P*-values were determined using the log-rank test

field, respectively; Figure 3B,D; n = 77). Thus, both CD8$^+$ TIL and T-bet$^+$ TIL are biased towards stromal localization in UCB.

Correlation between stromal TIL density and clinicopathological variables, including PD-L1 expression, was evaluated by χ^2 analysis. There was a positive correlation between CD8$_{ST}^+$ TIL density and age (*P* = .02), tumor size (*P* = .007), tumor stage (*P* < .001), nodal status (*P* = .002) and histological grade (*P* < .001). T-bet$_{ST}^+$ TIL cell density was positively associated with tumor stage (*P* < .001) and histological grade (*P* = .002; Table S3). Moreover, CD8$_{ST}^+$ TIL and T-bet$_{ST}^+$ TIL densities were both positively associated with PD-L1$^+$ TC (*P* < .0001 and *P* = .004; Figure 4A,C) and PD-L1$^+$ IC (both *P* < .0001; Figure 4B,D).

Kaplan-Meier analysis showed a negative association between CD8$_{ST}^+$ TIL density and OS (*P* < .001), but not RFS (*P* = .99; Figure S3a). Patients with high CD8$_{ST}^+$ TIL density had shorter OS than patients with low CD8$_{ST}^+$ TIL density (median, 48 vs 59 months). These results were identical to our previous results.[13] However, T-bet$_{ST}^+$ TIL density was not associated with OS or RFS (*P* > .05; Figure S3b).

Furthermore, we analyzed PD-L1 status and CD8$_{ST}^+$ TIL density in 128 patients with UCB. Patients were classified into three groups:

(I) PD-L1$^-$ TC and PD-L1$^-$ IC with low CD8$_{ST}^+$ TIL (n = 44); (II) either PD-L1$^+$ TC, PD-L1$^+$ IC or high CD8$_{ST}^+$ TIL (n = 66); and (III) PD-L1$^+$ TC and PD-L1$^+$ IC with high CD8$_{ST}^+$ TIL (n = 18). OS was significantly different (*P* = 0.002), and a trend towards a significant difference in recurrence was observed between groups I and III (*P* = .086; Figure S4).

3.5 | Associations between PD-1$^+$ TIL and PD-L1$^+$ IC, CD8$^+$ TIL and clinical outcomes

The known receptor for PD-L1 is PD-1, a key immunosuppressive marker expressed on exhausted TIL, which may be responsible for the poor outcome of higher CD8$_{ST}^+$ TIL density for UCB patients.[28] To test the hypothesis, distribution and clinical significance of PD-1$^+$ TIL were examined. PD-1$^+$ TIL were more frequent in ST than in INT (12 ± 1 and 4 ± 1 cells/field, respectively; Figure 5A,B; n = 82). Kaplan-Meier analysis showed a negative association between PD-1$_{ST}^+$ TIL density and OS (*P* = .0013; Figure 5C). Moreover, PD-1$_{ST}^+$ TIL density was significantly positively associated with PD-L1$^+$ IC (*P* < .0001; Figure 5D) and CD8$_{ST}^+$ TIL density (*P* < .0001; Figure 5E).

TABLE 2 Univariate and multivariate analyses of factors associated with mortality and recurrence in urothelial cell carcinoma of the bladder (n = 248)

	Overall survival				Recurrence			
		Multivariate				Multivariate		
Variable	Univariate	HR	95% CI	*P*-value	Univariate	HR	95% CI	*P*-value
Age, y (>60 vs ≤60)	0.803			NA	0.637			NA
Gender (female vs male)	0.434			NA	0.857			NA
Tumor size (>3 vs ≤3 cm)	**0.003**	1.35	0.698-2.604	0.37	0.101			NA
Multifocality (multifocal vs unifocal)	0.31			NA	0.39			NA
Tumor stage (T2-T4 vs Ta-T1)	**9.4×10^{-9}**	4.56	2.06-10.088	**1.8×10^{-4}**	**0.001**	1.665	0.87-3.186	0.124
Nodal status (N1-N2 vs N0)	**1.9×10^{-8}**	1.72	0.944-3.119	0.077	**0.002**	1.381	0.738-2.581	0.313
Metastasis status (M1 vs M0)	**0.02**	1.25	0.51-3.076	0.623	**0.029**	1.538	0.625-3.786	0.349
Histological grade (G3 vs G1-G2)	**0.002**	0.61	0.252-1.483	0.277	**0.041**	0.854	0.416-1.754	0.668
PD-L1 TC (positive vs negative)	**0.014**	0.98	0.565-1.714	0.954	**4.5×10^{-5}**	1.656	0.976-2.81	0.061
PD-L1 IC (positive vs negative)	**8×10^{-6}**	3.02	1.601-5.704	**0.001**	**0.002**	1.858	1.086-3.179	**0.024**

CI, confidence interval; HR, hazard ratio; IC, tumor-infiltrating immune cells; NA, not applicable; PD-L1, programmed death ligand-1; TC, tumor cells. Variables associated with mortality or recurrence in the univariate analysis were adopted as covariates the multivariate Cox regression analysis. Significant *P*-values are shown in bold. HR >1, increased risk; HR <1, decreased risk.

4 | DISCUSSION

Programmed death ligand-1/PD-1 signaling represents a mechanism of escape from antitumor immune responses.[14-16] Here, we characterized PD-L1 expression in TC and IC in the context of T-cell infiltration in all stages of resectable UCB. High PD-L1+ TC and PD-L1+ IC were associated with poorer OS and RFS in univariate analysis, and high PD-L1+ IC was an independent predictor of OS and RFS in multivariate analysis. Stromal infiltration of CD8+ TIL and PD-1+ TIL, but not T-bet+ TIL, was negatively associated with OS in univariate analysis. High PD-L1+ TC and PD-L1+ IC were associated with aggressive clinical features and high densities of stromal CD8+ TIL and T-bet+ TIL. Moreover, stromal PD-1+ TIL density was positively associated with PD-L1+ IC and stromal CD8+ TIL density. These data reflect the complicated tumor-host immune relationship and indicate the precise location of various immune cell populations that affect human tumor progression.

Programmed death ligand-1 is widely expressed in various solid tumors.[21] PD-L1+ TC has previously been detected in approximately 10%-40% of UCB specimens.[23,24] Based on the potential predictive value of PD-L1+ IC in patients with UCB receiving checkpoint blockade, attention has now switched to the associations between PD-L1+ IC and clinical parameters.[25] Bellmunt et al[25] found PD-L1 IC in 37% (33/89) of metastatic patients who received chemotherapy. By using a validated commercially available assay that is suitable for paraffin-embedded tissues and evaluating the proportion of PD-L1 expression,[17,27] we detected PD-L1+ TC in 23.4% (58/248) of treatment-naïve UCB specimens. PD-L1+ IC was present in 54.8% (136/248) of the same specimens, most notably at the invasive tumor-stromal margin. These discrepancies between studies could be as a result of tumor heterogeneity, variable cohort sizes, or different antibodies or IHC methodologies. Moreover, we found both PD-L1+ TC and PD-L1+ IC were associated with aggressive clinical features, such as larger tumor, poor differentiation and advanced UCB. Notably, PD-L1+ IC comprised diverse immune cell compositions with different intensities and variably sized aggregates in stroma of the same tissues, which made it more difficult to determine for intensities of PD-L1 expression by different cellular resources. We now know that the effects of PD-L1 can induce T-cell dysfunction through a variety of mechanisms, including the induction of T-cell apoptosis and exhaustion or regulation of IL-10 expression and Treg functions.[29-32] Therefore, the variable levels of PD-L1 expression in stromal cells might reflect their potentially different functions on tumor immune suppression.
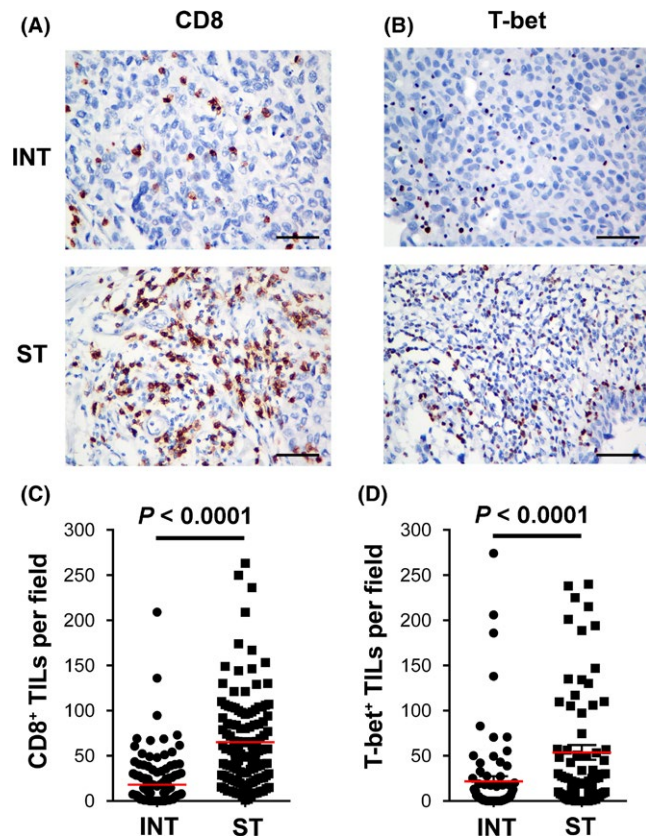
FIGURE 3 CD8+ tumor-infiltrating lymphocytes (TIL) and Th1 originated T-bet expression (T-bet+ TIL) are enriched in the stromal regions of urothelial cell carcinoma of the bladder (UCB). A,C, Representative examples of CD8+ TIL and T-bet+ TIL in the intratumoral (INT) and stromal regions (ST) of human UCB. Scale bar, 50 μm. B,D, CD8+ TIL and T-bet+ TIL densities were higher in ST than in matched INT (n = 128 and n = 77, respectively). Cell numbers were calculated as the cell count per field of view (×400)
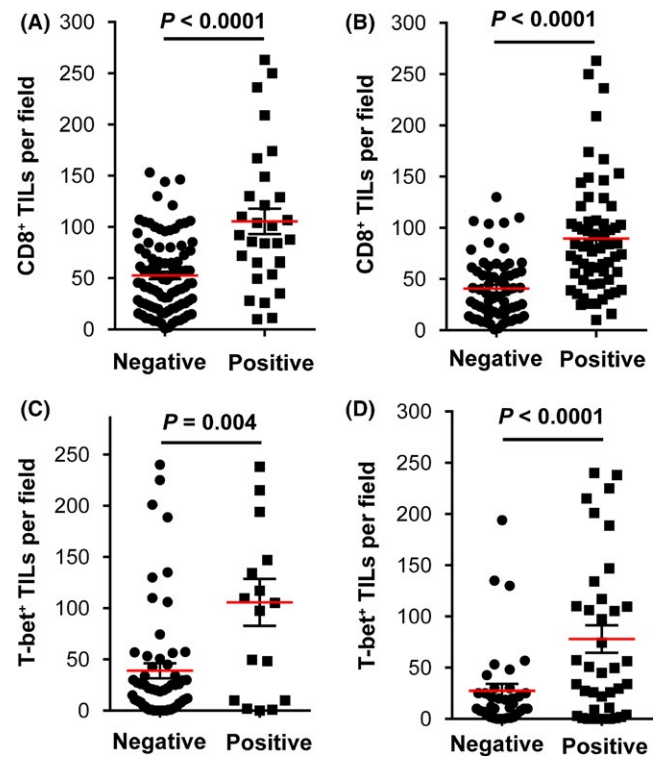

FIGURE 4 Quantitative assessment of stromal tumor-infiltrating lymphocytes (TIL) according to programmed death-ligand 1 (PD-L1) status. Both stromal CD8+ TIL (A,B) and T-bet+ TIL (C,D) were greater in patients with PD-L1+ tumor cells (TC) or PD-L1+ tumor-infiltrating immune cells (IC) than in PD-L1− TC or PD-L1− IC, respectively

Programmed death ligand-1 expression in the tumor microenvironment is thought to be regulated by tumor-associated stroma (adaptive immune resistance) and/or tumor cells (intrinsic immune resistance).[16] According to the "adaptive resistance" hypothesis, PD-L1 is upregulated by the effector molecule INF-γ in the tumor niche and, then, the PD pathway serves as a negative feedback mechanism to suppress tumor immunity.[21] As INF-γ is mainly secreted by effector T cells, we determined the density of CD8+ TIL and T-bet+ TIL in the INT and ST regions and explored their associations with PD-L1 expression. We observed higher CD8+ TIL and T-bet+ TIL densities in the stroma of patients with PD-L1+ TC or PD-L1+ IC than in PD-L1− TC or PD-L1− IC. Similarly, other studies have reported strong associations between PD-L1 and TIL in melanoma and gastric cancer.[33,34] In addition, tumor cells can express PD-L1 by intrinsic mechanisms, supporting our observation of a small fraction of UCB specimens with low CD8+ TIL density and high PD-L1+ TC and/or PD-L1+ IC (16%, 20/128). Moreover, several studies have demonstrated that loss of phosphatase and tensin homolog (PTEN) or overexpression of MYC or Cdk5 can directly upregulate PD-L1 in tumor cells.[35-37] However, the mechanisms of

oncogenic or induced PD-L1 expression in patients with UCB need further investigation.

Accumulating evidence has shown that high memory T-cell density is associated with longer OS in human cancers.[8] However, we found that a high stromal CD8+ T-cell density was associated with poorer OS in patients with resectable UCB;[13] this result was confirmed by the present study of a different cohort. Our results further indicated that in the stroma, CD8+ TIL density has strong positive association with PD-L1+ TC, PD-L1+ IC and PD-1+ TIL density. These results suggest an exhausted immune state occurs in the tumor stroma in UCB, a phenomenon also called immune privilege.[29] Immune privilege may take several forms to restrict antitumor function of infiltrating TIL, including regulation of the spatial distribution of T cells within tumors (intratumoral region and stroma region), impairment of T-cell activation or promotion of T-cell apoptosis by immune checkpoints (PD1/PD-L1, TIM-3, LAG-3 and IDO) or other cancer-associated stromal cell types (Treg, cancer-associated fibroblasts, and myelomonocytic cells).[29-32] In consistent with our results, a high fraction of FOXP3+ Treg/ CD8+ TIL was reported as a marker to predict an unfavorable prognosis in advanced UCB.[11] Moreover, a high ratio of CD68+ macrophages/CD3+ TIL was identified as a bad prognosis group among muscle-invasive bladder cancer.[38] Similarly, associations of PD-L1
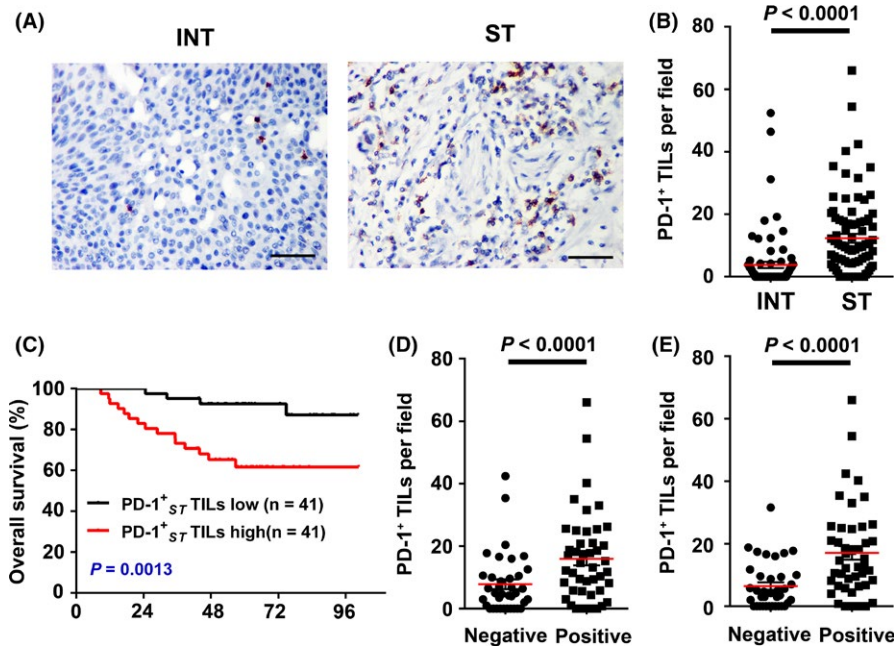
FIGURE 5 High stromal programmed death 1 (PD-1)[+] tumor-infiltrating lymphocyte (TIL) density predicted poor prognosis and was associated with programmed death-ligand 1 (PD-L1)[+] tumor-infiltrating immune cells (IC) and high stromal CD8^{+} TIL density. A, Representative examples of PD-1^{+} TIL in the intratumoral (INT) and stromal regions (ST) of human urothelial cell carcinoma of the bladder (UCB). Scale bar, 50 μm. B, PD-1^{+} TIL density was higher in ST than in matched INT (n = 82). Cell numbers were calculated as the cell count per field of view (×400). C, Kaplan-Meier overall curve for patients with UCB stratified by PD-1^{+} TIL. D,E, Stromal PD-1^{+} TIL were more frequent in patients with PD-L1^{+} IC or high stromal CD8^{+} TIL than in PD-L1^{−} IC or low stromal CD8^{+} TIL, respectively

expression and high stromal CD8^{+} TIL density with poor outcome have been reported in renal cell carcinoma.[39,40]

Whole genome transcriptomic analysis provides a novel way to identify tumor subtypes.[2,3,41] Moreover, Becht et al[42] found that different molecular subgroups of colorectal carcinoma had distinct immune signatures. In colorectal carcinoma, the microsatellite instability (MSI)-enriched subgroup with a good prognosis had high effector T-cell infiltration and low immunosuppressive myeloid cell density. In contrast, the mesenchymal subgroup with poor prognosis had high lymphocytic infiltration and high immunosuppressive myeloid cell density.[41] In bladder cancer, Robertson et al[2] identified five expression subtypes using multiple TCGA platforms. The luminal-infiltrated subtype and basal/squamous subtype showed medium to high expression of PD-L1 and other signs of immune infiltration and had the poorest prognosis.[2] Notably, the luminal-infiltrated subtype of metastatic or unresectable bladder cancer (corresponding to previous TCGA subtype II) has been reported to respond to anti-PD-L1 treatment.[43] These analyses indicate that combined analysis of immune signatures and molecular subtypes may yield a more nuanced understanding of the tumor-host immune relationship and provide further targets for immunotherapy.

This study has several limitations. First, this retrospective analysis assessed patients with treatment-naïve UCB only. We cannot compare our results with other studies of patients treated with BCG immunotherapy, chemotherapy or radiotherapy.[23-25] Second, high PD-L1 expression has previously been associated with both favorable and unfavorable outcomes in UCB.[21,23-25] This may be as

a result of the use of different PD-L1 antibodies and inconsistent evaluation of PD-L1 expression. Although the specificity of the anti-PD-L1 antibody used in this study has been confirmed in clinical trials in multiple cancers,[26,43] standardized criteria for evaluation of PD-L1 expression are urgently needed.

Our findings support the conclusion that PD-L1 is expressed by both TC and IC in all stages of resectable UCB, and PD-L1^{+} TC or IC are associated with inferior OS and RFS. Moreover, PD-L1^{+} TC and PD-L1^{+} IC were associated with high stromal CD8^{+} TIL and T-bet^{+} densities. Notably, we confirmed that stromal CD8^{+} TIL have predictive value for poor prognosis in UCB, which can be partly explained by coexistence of stromal CD8^{+} TIL and PD-1/PD-L1 expression. Continued clinical development of PD1/PD-L1 checkpoint inhibitors may be effective for all pathological stages of UCB.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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