PD-L1 Expression Reveals Significant Association With Squamous Differentiation in Upper Tract Urothelial Carcinoma

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ABSTRACT

Objectives: *Limited literature is available on the tumor microenvironment (TM) of upper tract urothelial carcinoma (UTUC). This study comprehensively reviews programmed death 1 receptor (PD-1)–positive and CD8+ tumor-infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) expression on tumor epithelium (TE).*

Methods: *Seventy-two nephroureterectomy specimens were analyzed for PD-L1, PD-1, and CD8. One percent or more tumor and lymphohistiocyte PD-L1 expression was considered positive. TIL density by H&E was scored semiquantitatively from 0 to 3, and CD8+ and PD-1+ TILs were quantified in hotspots.*

Results: *Of the cases, 37.5% demonstrated PD-L1+ on TE. PD-L1+ TE showed an association with pathologic stage (*P = *.01), squamous differentiation (SqD) (*P < *.001), TILs by H&E (*P = *.02), PD-1+ peritumoral TILs (*P = *.01), and PD-L1+ peritumoral lymphohistiocytes (*P = *.002). Finally, there was a significant difference in PD-1+ peritumoral TILs in cases with SqD vs no SqD (*P = *.03).*

Conclusions: *Aggressive UTUC is associated with a distinct TM. Furthermore, TM of UTUC-SqD was distinctly different from those with no SqD, warranting study in a larger cohort.*

Upper tract urothelial carcinoma (UTUC) is an uncommon but aggressive disease with up to 60% of cases being invasive at diagnosis.[1] It is thought to represent up to 5% of all urothelial cancers,[2] but the exact incidence is unclear as renal pelvic tumors are not distinguished from primary renal tumors in cancer statistics reporting.[3] In 2018, the estimated number of new ureteral tumors in the United States was about 3,800, and combined renal pelvic and kidney tumors accounted for about 65,000.[3]

Definitive treatment of UTUC has changed little over the past 50 years and consists of radical nephroureterectomy with resection of the intramural ureter. Unlike urinary bladder cancer (UBC), in which the majority (75%) of the tumors are superficial, most cases of UTUC are invasive at diagnosis.[4] The overall 5-year disease-specific mortality is calculated at approximately 25%, reflecting advanced stage at diagnosis, with a significant percentage (~28%) of survivors eventually developing recurrences.[5] Additional therapeutic options include partial ureterectomy, endoscopic management, and topical adjuvant therapies, with ongoing clinical trials on the utility of neoadjuvant chemotherapy proven to be effective in UBC such as methotrexate, vinblastine, doxorubicin, and cisplatin or a combination of gemcitabine and cisplatin.[6] There are, however, no prospective trials showing a survival benefit of this strategy in UTUC. Partial ureterectomy does not seem to show any significant difference in oncologic outcomes compared with radical nephroureterectomy in a large retrospective study,[7] while endoscopic

management has shown 5-year local recurrences as high as 50%.[8]

While UTUC and UBC share many morphologic and genetic similarities, gene expression differences have been found when these are separated by pathologic T stage. Interestingly, the differentially expressed genes appear to have immunologic functions and were found to be involved in the tumor necrosis factor (TNF) and hepatocyte growth factor (HGF) pathways.[9] These new findings may have implications in response to immune-mediated therapy. Recently, immunotherapy with checkpoint inhibitors has come to the forefront of cancer therapy in a big way, highlighting the vital role of the tumor microenvironment (TM).[10-12] One of the targets of cancer immunotherapy is the programmed death 1 receptor (PD-1)/programmed death ligand 1 (PD-L1) signaling pathway. After immunologic activation, PD-1 is expressed on the surface of tumor-infiltrating lymphocytes (TILs), including activated T-lymphocytes, B-lymphocytes, and histiocytes. PD-L1 is limitedly expressed in normal cells and interacts with its receptor, PD-1, to protect healthy cells from excessive inflammatory or autoimmune responses. When PD-L1 is expressed on tumor cells, this very interaction with PD-1 serves as a mechanism of immune escape for tumor cells by inhibiting the activated T-lymphocytes.[13] The interaction of PD-L1 on tumor cells and its receptor, PD-1, is thus an important mechanism leading to enhanced tumor cell growth and is associated with poor prognosis in many solid organ malignancies.

Various PD-1 and PD-L1 inhibitors have shown favorable results for patients with urothelial carcinoma (UC),[14-20] with now five US Food and Drug Administration (FDA)–approved PD-1/PD-L1 inhibitors for UC.[21] Immunohistochemistry (IHC) for PD-L1 is a useful predictive marker for PD-1/PD-L1 inhibitors in certain malignancies such as non–small cell lung carcinoma, but this association has not been found in UC. All of the current clinical trials in UC have shown objective responses in all patients regardless of PD-L1 status, with the exception of studies for durvalumab and pembrolizumab, where a higher objective response was noted in PD-L1 high tumors.[14,19] Formal clinical trials focusing on UTUC have not yet been performed. Some of the PD-1/PD-L1 clinical trials included patients with UTUC and also noted outcome data on this subgroup of patients.[14-17] However, UTUC cases accounted for less than one-third of patients in such trials. While some noted comparable response rates in UTUC vs UBC cases, atezolizumab, a PD-L1 inhibitor, showed a higher objective response rate of 39% in UTUC compared with 17% in UBC.[15]

In summary, in light of the increasing role of immunotherapy combined with new data highlighting the molecular pathway differences between UTUC and UBC, it is important to understand the TM for UTUC. Several studies have evaluated the TM of UBC through IHC,[22-29] with only few studies characterizing UTUC.[30-34] Hence, in this retrospective study, we performed a comprehensive clinical and histologic review of UTUC, with detailed analysis of PD-L1 expression in various tumor compartments and assessment of tumor-infiltrating immune cells with respect to CD8 and PD-1 expression.

Materials and Methods

Case Selection

Following approval from the institutional review board of the Hospital of the University of Pennsylvania, urology and pathology archives were used to identify nephroureterectomy specimens of UTUC from January 2000 through December 2015. Archived slides were reexamined and histologic diagnoses and staging were confirmed as based on the most recent World Health Organization classification of tumors of the urinary system by a genitourinary pathologist (P.L.). Clinicopathologic variables, including patient age, sex, race, tumor size, tumor grade, stage, lymph node status, margin status, presence of carcinoma in situ, and patient outcomes, were obtained from the electronic medical record, pathology report, and slide review.

Immunohistochemistry

Selected whole sections of paraffin-embedded, formalin-fixed tissue were stained for antibodies against PD-L1, PD-1, and CD8. Staining was performed on a Leica Bond-IIITM instrument using the Bond Polymer Refine Detection System (DS9800; Leica Microsystems). Heat-induced epitope retrieval was done for 20 minutes with either ER1 or ER2 solution (Epitope Retrieval 1 [ER1] AR9961 or Epitope Retrieval 2 [ER2] AR9640; Leica Microsystems). The various clones and conditions used for each of the epitopes are as follows: PD-1 Abcam clone NAT105 (catalog ab52587) at a 1:40 dilution with ER1 solution, PD-L1 Cell Signalling clone E1J2J (catalog 15165BF) at a dilution of 1:2,000 with ER2 solution, and CD8 Dako clone C8/144B (catalog M7103) at a dilution of 1:40 with ER1 solution.

PD-L1 Evaluation

Expression of PD-L1 was assessed in three compartments: tumor epithelium (TE), lymphohistiocytic clusters within papillary cores (LH-PCs), and peritumoral lymphohistiocytic clusters (LH-PTs). Lymphohistiocytic

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clusters are inflammatory clusters of intimately admixed lymphocytes and histiocytes. These cells were combined in our evaluation of PD-L1, as it is difficult to subtract the lymphocytes from histiocytes in the absence of double staining for both. The percent staining of LH-PCs and LH-PTs, if present, was recorded for each tumor. PD-L1 was considered positive in the TE component when 1% or more partial or complete membranous staining was identified. Since there are no currently established criteria for evaluating PD-L1 on tumor cells, we used a minimum accepted cutoff of 1% or more. The intensity of PD-L1 staining in the three compartments was also scored as absent (0), weak (1), moderate (2), and strong (3). To normalize the data for comparison of PD-L1 staining between cases, an H-score was calculated for each compartment using the following formula: intensity of staining × percentage of cells staining.

CD8 and PD-1 TILs

The degree of TILs was semiqualitatively assessed by H&E-stained slides, and the intensity was scored as absent (0), mild (1), moderate (2), or severe (3). CD8+ and PD-1+ lymphocytes were quantified manually in hotspot areas (an average number of positive cells/10 high-power fields) in three tumor compartments: peritumoral, intraepithelial, and papillary cores. Two independent pathologists (A.G.A. and P.L.) assessed all histologic parameters without any prior knowledge of the clinical data.

Statistical Analysis

Differences and associations between various categorical variables were assessed using the Pearson χ^2 square test, while differences in continuous variables were assessed using the nonparametric Wilcoxon rank-sum test. *P* values less than .05 were considered significant. Overall and disease-free survival were estimated using the Kaplan-Meier method. The potential effect of different histologic parameters on the overall and disease-free survival was evaluated using the Cox proportional hazard regression model. The continuous variables were entered into the regression model once as a continuous variable and then as a categorical variable. The 50th and 75th percentiles were used to define the cutoff points in converting the continuous variables to categorical ones. Statistical analysis was performed using Stata/SE13.1 (StataCorp).

Results

A total of 72 nephroureterectomy specimens were included in the study. The mean patient age was 68 years (range, 31-96 years), with an equal number of male and female patients. There were 60 cases of high-grade UC and 12 cases of low-grade UC. Pathologic stage was distributed as follows: pTa, n = 25; pT1, n = 13; pT2, n = 7; pT3, n = 25; and pT4, n = 2. Variable amounts of squamous differentiation were seen in 14 cases. Other variant morphologies include one case displaying both glandular and neuroendocrine differentiation, two cases with inverted growth pattern, and one case with a lymphoepithelioma-like morphology. Other clinicopathologic features are summarized in **Table 1**. None of the cases were treated with neoadjuvant therapy.

Overall PD-L1 Expression

PD-L1 staining was positive in the TE component in 27 (37.5%), in the LH-PC component in 15 (20.8%), and in the LH-PTs in 29 (40.3%) of the included cases (Table 1). Only four cases showed PD-L1+ staining in all three compartments. The percentage of TE component that was PD-L1 positive was widely varied across the included cases. PD-L1+ TE staining ranged from 1% to 90% (median, 5%), with just one-third (8/27, 29.9%) of PD-L1+ TE cases showing more than 5% staining (range, 10%-90%). The breakdown of positive cases by PD-L1 TE percent staining is as follows: n = 19 with 1% to 5%, n = 6 with 10% to 50%, and n = 2 with more than 50%. Among PD-L1+ TE cases, six (29.3%) were also PD-L1+ in LH-PCs and 17 (65.4%) were PD-L1+ in LH-PTs. There was a significant association in PD-L1 TE expression with regard to PD-L1 in LH-PTs (*P* = .002) when LH-PTs were analyzed as a categorical value **Figure 1**. However, this was not the case for PD-L1 in LH-PCs (*P* = .533).

Invasive Front vs Noninvasive Tumor

We decided to further study the expression of PD-L1 in both noninvasive (NI) and invasive (INV) front of the tumor. This was thought to be important as the INV front is considered more aggressive, and we hypothesized that the TM at the INV front may be different from the more indolent NI cell population. Of the 15 cases with positive PD-L1 staining in the TE component, three cases displayed differences in PD-L1+ TE in the NI vs INV components of the tumor. Two of these cases showed higher PD-L1+ TE in the INV vs NI component (100% and 90% vs 1% and 5%, respectively), while the other showed less PD-L1+ TE in the INV vs NI component (5% vs 10%).

Stage and Immune Microenvironment

Figure 2 provides a synopsis of the distribution of PD-L1 by tumor component and pathologic stage.

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Table 1
Summary of Clinicopathologic Features[a]

Parameter	Value
Age, mean (range), y	68 (31-96)
Sex	
Male	36 (50.0)
Female	36 (50.0)
Race	
Non-AA	69 (95.8)
AA	3 (4.2)
Tumor location	
Renal pelvis	36 (50.0)
Ureter	12 (16.7)
Both	24 (33.3)
Histologic grade	
Low grade	12 (16.7)
High grade	60 (83.3)
Pathologic stage	
Ta	25 (34.7)
T1	13 (18.1)
T2	7 (9.7)
T3	25 (34.7)
T4	2 (2.8)
Squamous differentiation	
Yes	14 (19.7)
No	57 (80.3)
Tumor size, mean (range), cm	3.8 (0.6-12.5)
Lymph node status	
N0	7 (9.7)
N+	6 (8.3)
Nx	59 (81.9)
Margin status	
Negative	60 (84.5)
Positive	11 (15.5)
Lymphovascular invasion	
Negative	23 (32.0)
Positive	17 (23.6)
Suspicious	6 (8.3)
Unknown	26 (36.1)
Carcinoma in situ	
Present	22 (30.5)
Absent	48 (66.7)
Unknown	2 (2.8)
Disease status	
Unknown	10 (13.9)
NED	32 (44.4)
AWD	1 (1.4)
DOD	18 (25.0)
DOC	11 (15.3)
TIL intensity by H&E	
Absent	7 (9.8)
Mild	37 (52.1)
Moderate	18 (25.4)
Marked	9 (12.7)
PD-L1 TE	
Positive	27 (37.5)
Negative	44 (61.1)
Not applicable	1 (1.4)
PD-L1 LH-PCs	
Positive	15 (20.8)
Negative	51 (70.8)
Not applicable	6 (8.3)
PD-L1 LH-PTs	
Positive	29 (40.3)
Negative	41 (56.9)
Not applicable	2 (2.8)

AA, African American; AWD, alive with disease; DOC, died of other cause; DOD, died of disease; LH-PCs, lymphohistiocytes in papillary cores; LH-PTs, peritumoral lymphohistiocytes; NED, no evidence of disease; PD-L1, programmed death ligand 1; TE, tumor epithelium; TIL, tumor-infiltrating lymphocyte.
[a]Values are presented as number (%) unless otherwise indicated.

PD-L1+ TE showed a significant association with pathologic stage ($P = .01$) when comparing non-muscle-invasive (pT <2) vs muscle-invasive (≥pT2) tumors, with non-muscle-invasive tumors showing significantly less PD-L1+ TE expression **Table 2**. This association remained significant when analyzing the extent of PD-L1 TE positivity through PD-L1 TE H-scores (0.58% ± 1.53% for pT <2 vs 18.54% ± 42.76% for ≥pT2, $P = .013$; **Figure 3**).

PD-L1 TE Expression and TILs

There was also a significant association in the intensity of TILs assessed by H&E with respect to PD-L1 TE expression ($P = .02$), with PD-L1+ TE cases displaying more intense TILs. PD-L1+ TE cases also showed a significant association with average PD-1+ PT lymphocytes ($P = .01$) and PD-L1+ LH-PTs ($P = .002$). However, the extent of PD-L1 TE expression did not correlate with the intensity of TILs or PD-1+ PT lymphocytes when assessing PD-L1 TE as a continuous value using H-scores (data not shown). No other significant associations were identified with PD-L1+ TE expression and other clinicopathologic parameters such as age, sex, tumor size, histologic grade, average CD8+ TILs in various compartments, and average PD-1+ TILs in TE or PC (Table 2).

Histologic Subtype and Immune Microenvironment

Finally, PD-L1 TE expression was identified in all cases (14/14, 100%) of UTUC with squamous differentiation (SqD) and in 13 (22.8%) of 57 cases without SqD. This difference was strongly significant ($P < .001$) (Table 2), and the extent of PD-L1 TE expression as measured by PD-L1 TE H-scores was significantly different in tumors with SqD compared with cases without SqD (40.07% ± 60.51% vs 1.59% ± 5.82%; $P < .0001$; **Figure 4**). In 12 (85.7%) of the 14 cases with SqD, it was predominantly the squamous component of the tumor that expressed PD-L1 diffusely. The other two cases showed

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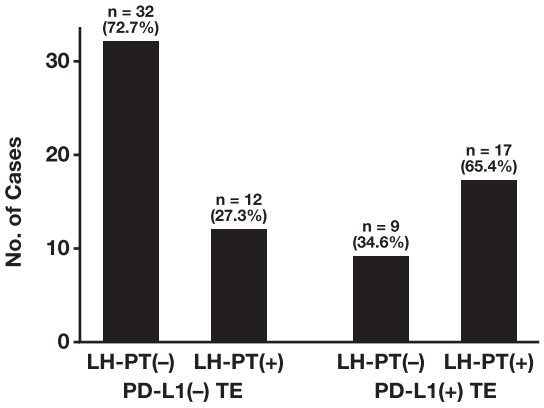

Figure 1 Distribution of cases with programmed death ligand 1 (PD-L1) expression in both tumor epithelium (TE) and peritumoral lymphohistiocytes (LH-PTs). $P = .002$.

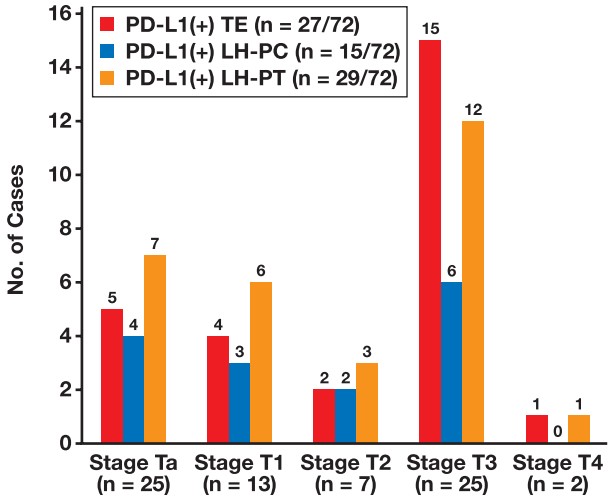

Figure 2 Summary of programmed death ligand 1 (PD-L1) expression by tumor component and stage. LH-PC, lymphohistiocytes in papillary cores; LH-PT, peritumoral lymphohistiocytes; TE, tumor epithelium.

PD-L1 expression in the classic urothelial areas. **Image 1** shows representative pictures of PD-L1 staining in cases with SqD. The only significant difference in PD-L1+ TE cases with and without SqD was the mean PD-L1 TE H-score, with PD-L1+ TE cases without SqD showing higher mean H-scores **Table 3**. No other significant differences were identified in PD-L1+ TE cases with and without SqD, as summarized in Table 3. However, tumors with SqD (n = 14) vs no SqD (n = 57), regardless of PD-L1 TE expression, showed a significant difference in average PD-1+ PT lymphocytes ($P = .03$). Other components of the TM, including CD8+ TILs, were not different in tumors with and without SqD regardless of PD-L1 TE status **Table 4**. **Image 2** shows representative pictures of CD8 and PD-1 staining in the TM.

Outcome Analysis

Partial follow-up data were available for 69 cases, with a mean follow-up interval of 98.7 months (range, 0.9-1,316 months). Disease status for these cases is summarized in Table 1. Mortality data were available for 64 patients, of whom 18 (28%) died of disease. Twenty-five (39%) patients had disease recurrence/progression, and of these, 11 (44%) of 25 developed metastatic disease (lung, liver, lymph nodes, bone), and 14 (56%) of 25 developed recurrence in the lower tract or opposite ureter (one case). There were 55 cases with complete follow-up data that could be used for statistical analysis of disease-free and overall survivals. No significant difference in disease-free survival, overall survival, and cancer-specific mortality was seen in PD-L1+ TE cases with and without SqD (Table 3 and **Table 5**). There was also no difference in overall survival in cases with SqD vs no SqD regardless of PD-L1 TE status (Tables 4 and 5). In addition, there was no difference in disease-free and overall survival when analyzing TILs by CD8, PD-1, and PD-L1 expression both as categorical (data not shown) and continuous variables (Table 5). The only significant finding on outcome analysis was a shorter disease-free survival for patients with pathologic stage T3 tumors (Table 5).

Discussion

With immunotherapy playing an ever-increasing role in the treatment of cancer, an understanding of the TM with respect to PD-L1 expression and TIL phenotype has become essential. The TM of UBC has been extensively characterized,[22-29] but to date, only a few studies characterizing UTUC have been published.[30-34] To our knowledge, none of these studies so far have performed a comprehensive review of both CD8+/PD-1+ TILs and PD-L1 expression on tumor cells and tumor-infiltrating lymphohistiocytes in various tumor compartments.

To our knowledge, only three studies have investigated the expression of PD-L1 in UTUC. A study by Krabbe et al[32] examined PD-1 and PD-L1 expression in patients with high-grade UC. They used tissue microarrays and the PD-L1 IHC clone E1L3N with a cutoff of 1% for being considered positive. In their cohort of 423 cases, 26.2% showed PD-L1 expression, which was also predictive of favorable outcomes in cases of organ-confined disease. On the other hand, they showed that PD-1 expression in TILs was associated with worse outcomes. Zhang et al[34] also investigated PD-L1 E1L3N expression in UTUC but used whole tissue sections with a positive cutoff of 5%. In their study, 12.3% of 162 cases expressed PD-L1, and PD-L1 was predictive of shorter cancer-specific survival. Cases with high PD-L1 in

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Table 2
Analysis of PD-L1 Tumor Epithelial Expression and Association With Various Clinicopathologic Parameters

Characteristic	PD-L1– TE (n = 44)	PD-L1+ TE (n = 27)	*P* Value[a]
Sex, No. (%)			.74
Male	21 (47.7)	14 (51.9)	
Female	23 (52.3)	13 (48.1)	
Age, mean ± SD, y	67.64 ± 12.21	66.96 ± 12.00	.95
Tumor size, mean ± SD, cm	3.53 ± 2.25	4.76 ± 2.92	.07
Pathologic stage (stages <2 vs ≥2), No. (%)			**.01**
<T2	28 (63.6)	9 (33.3)	
≥T2	16 (36.4)	18 (66.7)	
H&E TIL intensity, No. (%)			**.02**
None	19 (43.2)	3 (11.1)	
Mild	15 (34.1)	10 (37.1)	
Moderate	7 (15.9)	8 (29.6)	
Marked	3 (6.8)	6 (22.2)	
Squamous differentiation, No. (%)			**<.001**
Yes	0 (0)	14 (51.9)	
No	44 (100)	13 (48.1)	
Average PD-1+ lymphocytes, mean ± SD			
Peritumoral	29.68 ± 28.67	50.36 ± 30.74	**.01**
Tumor epithelial	2.15 ± 5.80	4.07 ± 0.47	.12
Papillary cores	12.07 ± 20.36	15.08 ± 20.86	.30
Average PD-L1 lymphohistiocytes, mean ± SD			
Peritumoral	0.27 ± 0.45	0.65 ± 0.48	**.002**
Papillary cores	0.20 ± 0.41	0.27 ± 0.46	.54
Average CD8+ lymphocytes, mean ± SD			
Peritumoral	64.06 ± 20.56	73.66 ± 19.75	.11
Tumor epithelial	6.04 ± 13.19	10.67 ± 16.78	.08
Papillary cores	31.92 ± 30.02	43.19 ± 37.11	.16

PD-1, programmed death 1 receptor; PD-L1, programmed death ligand 1; TE, tumor epithelium; TIL, tumor-infiltrating lymphocyte.
[a]Bold values are significant ($P < .05$).

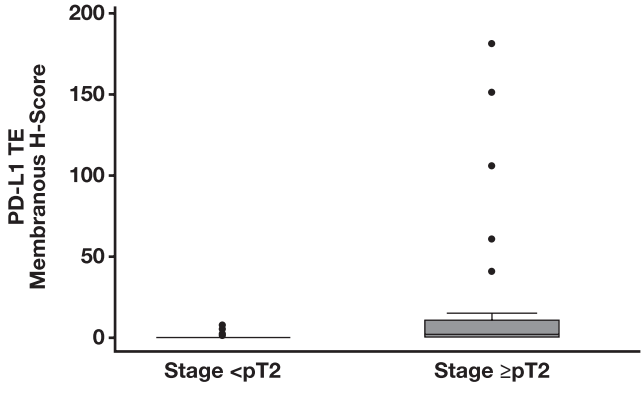

Figure 3 Extent of programmed death ligand 1 (PD-L1) tumor epithelium (TE) expression in relation to pathologic tumor stage. $P = .01$.

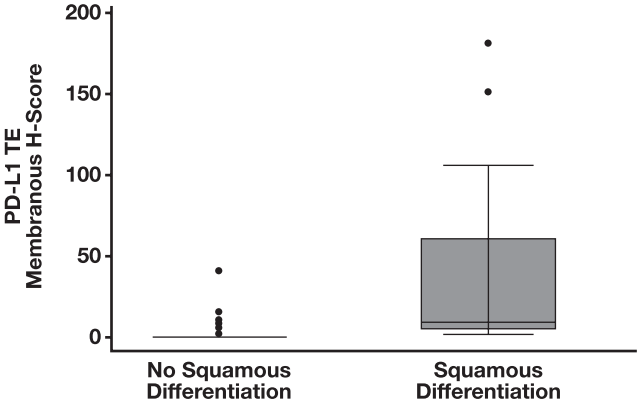

Figure 4 Extent of programmed death ligand 1 (PD-L1) tumor epithelium (TE) expression in relation to squamous differentiation. $P < .0001$.

tumor-infiltrating mononuclear cells were noted to be associated with higher PD-L1 on tumor cells. Both studies by Krabbe et al[32] and Zhang et al[34] did not state whether their cases included UTUC with variant morphologies or if there was any associations with PD-L1 expression. Finally, Skala et al[33] used PD-L1 clone 5H1 with a positive cutoff of 5% on whole tissue sections of 149 UTUC cases. In this study, 23.5% of cases were positive for PD-L1. PD-L1 expression was associated with higher grade, stage, and lymphovascular invasion, with 50% or more PD-L1 being significantly associated with cancer-specific mortality. Importantly, they noted an association of PD-L1 with divergent histology with the presence of sarcomatoid and squamous components displaying diffuse PD-L1 expression, although this association was not statistically significant.

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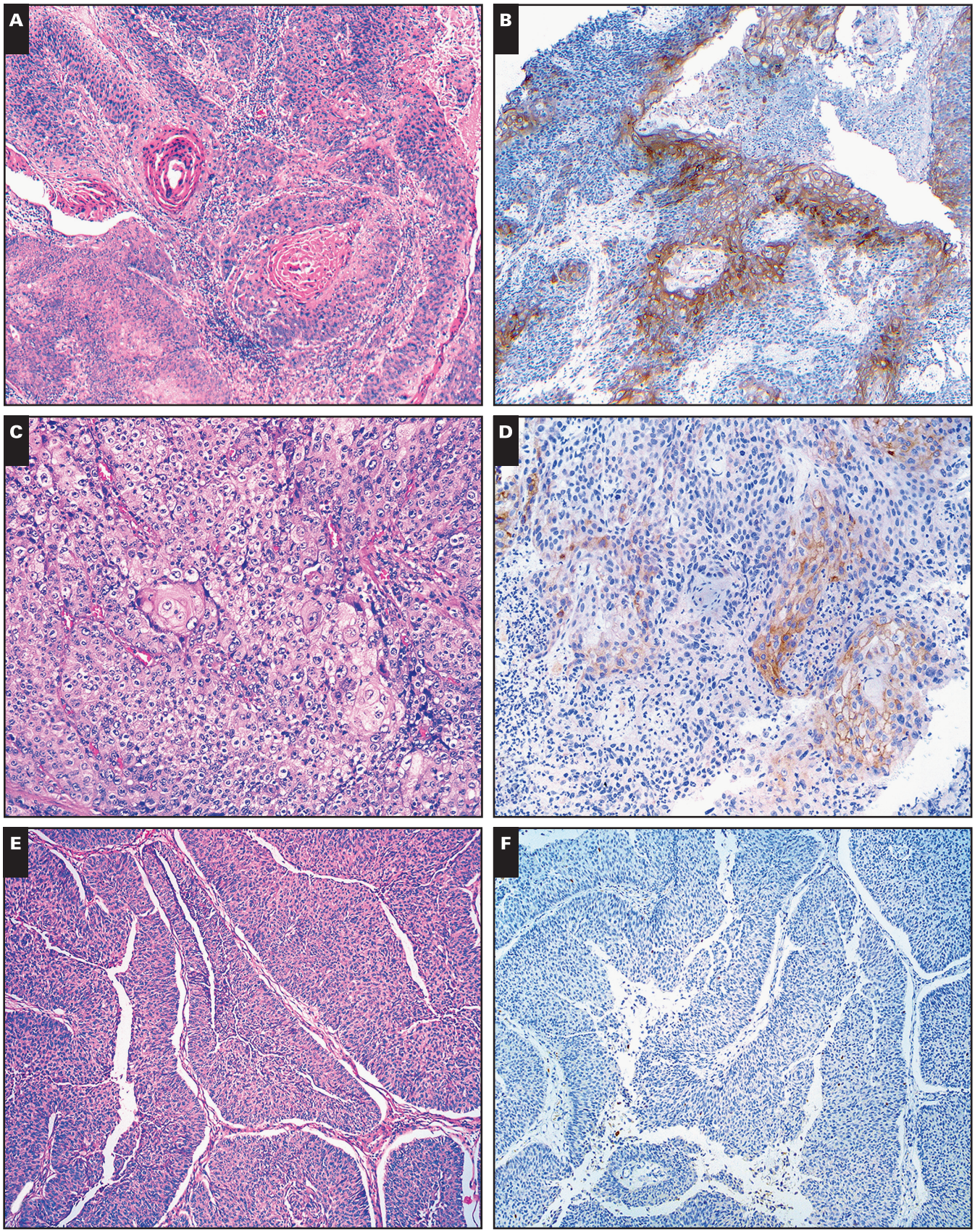

Image 1 Programmed death ligand 1 (PD-L1) tumor epithelial expression in select upper tract urothelial cases: (**A**, **B**) 3+ membranous expression in 50% of tumor primarily in squamous component, (**C**, **D**) 1+ membranous expression in 5% of tumor primarily in squamous component, and (**E**, **F**) negative (0%) PD-L1 expression in noninvasive papillary high-grade urothelial carcinoma. (**A**, H&E, ×50; **B**, PD-L1, ×50; **C**, H&E, ×200; **D**, PD-L1, ×200; **E**, H&E, ×100; **F**, PD-L1, ×100.)

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Table 3
Analysis of Clinicopathologic Features and Survival in PD-L1+ Tumor Epithelial Cases With and Without Squamous Differentiation

Characteristic	PD-L1+ TE Without SqD (n = 13)	PD-L1+ TE With SqD (n = 14)	*P* Value[a]
Sex, No. (%)			
Male	5 (38.5)	9 (64.3)	.18
Female	8 (61.5)	5 (35.7)	
Age, mean ± SD, y	69.38 ± 8.46	64.71 ± 14.52	.59
Tumor size, mean ± SD, cm	5.78 ± 3.34	3.81 ± 2.17	.08
Pathologic stage, No. (%)			
pTa	2 (15.4)	3 (21.4)	
pT1 and pT2	4 (30.8)	2 (14.3)	.58
pT3 and pT4	7 (53.8)	9 (64.3)	
PD-L1+ H-score, mean ± SD			
Tumor epithelial	67.67 ± 10.21	45.92 ± 63.83	**.01**
Peritumoral	7.5 ± 21.94	9.64 ± 13.48	.12
Average PD-1+ lymphocytes, mean ± SD			
Tumor epithelial	4.03 ± 7.63	4.11 ± 7.60	.71
Papillary cores	14.76 ± 24.98	18.17 ± 25.78	.69
Peritumoral	54.90 ± 40.74	63.12 ± 36.58	.63
Average CD8+ lymphocytes, mean ± SD			
Tumor epithelial	9.49 ± 10.76	12.84 ± 24.80	.38
Papillary cores	47.57 ± 46.06	49.32 ± 39.68	.97
Peritumoral	90.41 ± 23.43	82.29 ± 19.10	.33
Survival, mean ± SD, mo	46.68 ± 64.68	15.13 ± 13.50	.09
Cancer-specific mortality, No. (%)			
Dead due to cancer	5 (38.4)	11 (78.6)	
Alive or dead due to other causes	4 (30.8)	3 (21.4)	.24
Unknown	4 (30.8)	0 (0)	

PD-1, programmed death 1 receptor; PD-L1, programmed death ligand 1; SqD, squamous differentiation; TE, tumor epithelium.
[a]Bold value is significant ($P < .05$).

PD-L1 TE expression in UBC seems to depend on the type of antibody used and a set cutoff point. This was demonstrated by Davick et al,[35] who used four different IHC methods where the expression of PD-L1 in TE ranged from 13.3% to 46.7% (n = 180) using a cutoff of 1%. This variation was also noted in a larger study by Tretiakova et al.[36] This latter study on UBC included 20 cases of variant morphologies (only two with squamous differentiation) and did not find any significant differences compared with conventional UBC.[36] Other UBC studies demonstrate 17%, 20%, and 28% of cases showing positive PD-L1 TE expression; however, these studies do not mention whether any variant morphologies were part of their cohorts.[23,25,26] Although different antibodies were used in these studies compared with ours, these ranges are comparable to our results in UTUC with 37.5% of PD-L1+ TE cases.

Two recent studies on UBC by Davick et al[35] and Udager et al[37] demonstrated more frequent PD-L1 expression in urinary bladder squamous cell carcinoma (UBSCC). Davick et al[35] demonstrated a statistically significant difference in UBSCC PD-L1 expression compared with conventional UBC (70% vs 43%, $P = .02$).[35] The study by Udager et al[37] also revealed a high percentage of UBSCC PD-L1 positivity (64.7%, n = 11/17). Furthermore, some cases revealed a more prominent expression of PD-L1 at the leading invasive tumor front.[37] This study, however, did not include cases of conventional UBC. Although these two studies involve pure squamous cell carcinomas of the urinary bladder, these findings, combined with our findings in UTUC, lend support to the possibility that any UC with a squamous component is more likely to express PD-L1 compared with conventional UC.

A few of the clinical trials on PD-1/PD-L1 inhibitors included a subgroup analysis of UTUC compared with UBC. For pembrolizumab, which is an immunotherapy agent targeting PD-1, UTUC cases showed similar objective response rates compared with UBC (22% vs 28%).[14] Avelumab, which targets PD-L1, also showed similar objective response rates between UTUC and UBC (11% vs 18%).[17] Both trials, however, had less than 25% of total cases that were from the upper tract. On the other hand, atezolizumab, which is another PD-L1 inhibitor, revealed higher objective response rates in UTUC (39%) compared with UBC (17%).[15] UTUC cases comprised 28% of the cohort for this study. Hence, these preliminary data, combined with knowledge of the difference in the mutational profiles of UTUC compared with UBC,[9,38] raise the possibility that UTUC should be treated differently with regard to select immunotherapeutic agents. Whether PD-L1 expression by IHC or the phenotype of the TM

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Table 4
Analysis of Squamous Differentiation and the Tumor Microenvironment

Characteristic	No Squamous Differentiation (n = 58)	Squamous Differentiation (n = 14)	*P* Value[a]
H&E TIL intensity, No. (%)			.30
None	20 (34.5)	2 (14.3)	
Mild	21 (36.2)	4 (28.6)	
Moderate	10 (17.3)	5 (35.7)	
Marked	6 (10.3)	3 (21.4)	
Not reported	1 (1.7)	0 (0)	
Average PD-1+ lymphocytes, mean ± SD			
Peritumoral	25.95 ± 19.81	39.12 ± 18.18	**.03**
Tumor epithelial	2.59 ± 6.25	4.11 ± 7.60	.43
Papillary cores	10.55 ± 14.75	14.63 ± 15.58	.52
Average PD-L1 H-score, mean ± SD			
Tumor epithelial	1.6 ± 1.5	40.7 ± 31.6	**<.001**
LH-PCs	5.17 ± 14.73	7.14 ± 12.66	.62
LH-PTs	12.6 ± 37.98	9.7 ± 13.43	.78
PD-L1 LH-PCs, No. (%)			.43
None	43 (74.1)	8 (56.1)	
Positive	11 (19.0)	4 (28.6)	
Not reported	4 (6.9)	2 (14.3)	
PD-L1 LH-PTs, No. (%)			.11
None	36 (62.1)	5 (35.7)	
Positive	20 (34.5)	9 (64.3)	
Not reported	2 (3.4)	0 (0)	
Average CD8+ lymphocytes, mean ± SD			
Tumor epithelial	6.4 ± 10.48	10.05 ± 16.15	.27
Papillary cores	26.70 ± 21.46	31.59 ± 20.07	.49
Peritumoral	47.01 ± 12.96	47.70 ± 11.03	.72
Survival, mean ± SD, mo	56.6 ± 12.4	33.7 ± 41.99	.11

LH-PCs, lymphohistiocytes in papillary cores; LH-PTs, peritumoral lymphohistiocytes; PD-1, programmed death 1 receptor; PD-L1, programmed death ligand 1; TE, tumor epithelium; TIL, tumor-infiltrating lymphocyte.
[a]Bold values are significant ($P < .05$).

in UTUC translates to an improved clinical response to PD-1/PD-L1 inhibitors is something that needs additional investigation.

In the present study, we observed PD-L1 expression in both tumor cells and tumor-infiltrating lymphohistiocytes. Of our cases, 37.5% showed PD-L1 expression in tumor cells, with 20.8% showing expression in lymphohistiocytes within papillary cores and 40.3% in peritumoral lymphohistiocytes. PD-L1 was expressed on tumor cells in all cases with squamous differentiation (n = 14), with 12 of 14 of these cases showing isolated PD-L1 expression in the squamous component of the tumor. Our observations are similar to those noted by Skala et al,[33] although we found PD-L1 expression and squamous differentiation to be strongly statistically significant ($P < .001$). When comparing tumors with and without squamous differentiation, there was a statistically significant difference in the average number of PD-1+ peritumoral lymphocytes with a higher average in tumors with squamous differentiation. We also found that PD-L1+ tumor expression was associated with higher TILs as assessed by H&E. More specifically, PD-1+ and PD-L1+ peritumoral lymphohistiocytes both correlated with PD-L1+ TE expression.

Our findings suggest a unique immunogenic environment in UTUC with squamous differentiation. In fact, some studies have observed high PD-L1 expression in squamous cell carcinomas of the head and neck and anal region.[39,40] These authors noted different patterns of PD-L1 tumor expression from diffuse to localized within the tumor cell–immune cell interface, consistent with the idea of an adaptive vs a constitutive mechanism for PD-L1 expression.[12] The adaptive pattern was first described by Taube et al[41] in melanoma, where it was noted that TILs likely induce the expression of tumor PD-L1 with cytokine secretion. This pattern of expression is geographically isolated to the tumor cell–immune cell interface. On the other hand, constitutive PD-L1 expression is independent of the immune cell infiltrate, wherein the driving force is thought to be due to an intrinsic factor such as the activation of oncogenic pathways that lead to diffuse PD-L1 overexpression.[12] In our study, PD-L1 was mostly observed to show diffuse expression in the squamous component of UTUC, which would be suggestive of the constitutive mechanism of PD-L1 expression. However, as we also observed increased PD-1+ peritumoral lymphocytes in tumors with squamous differentiation, an

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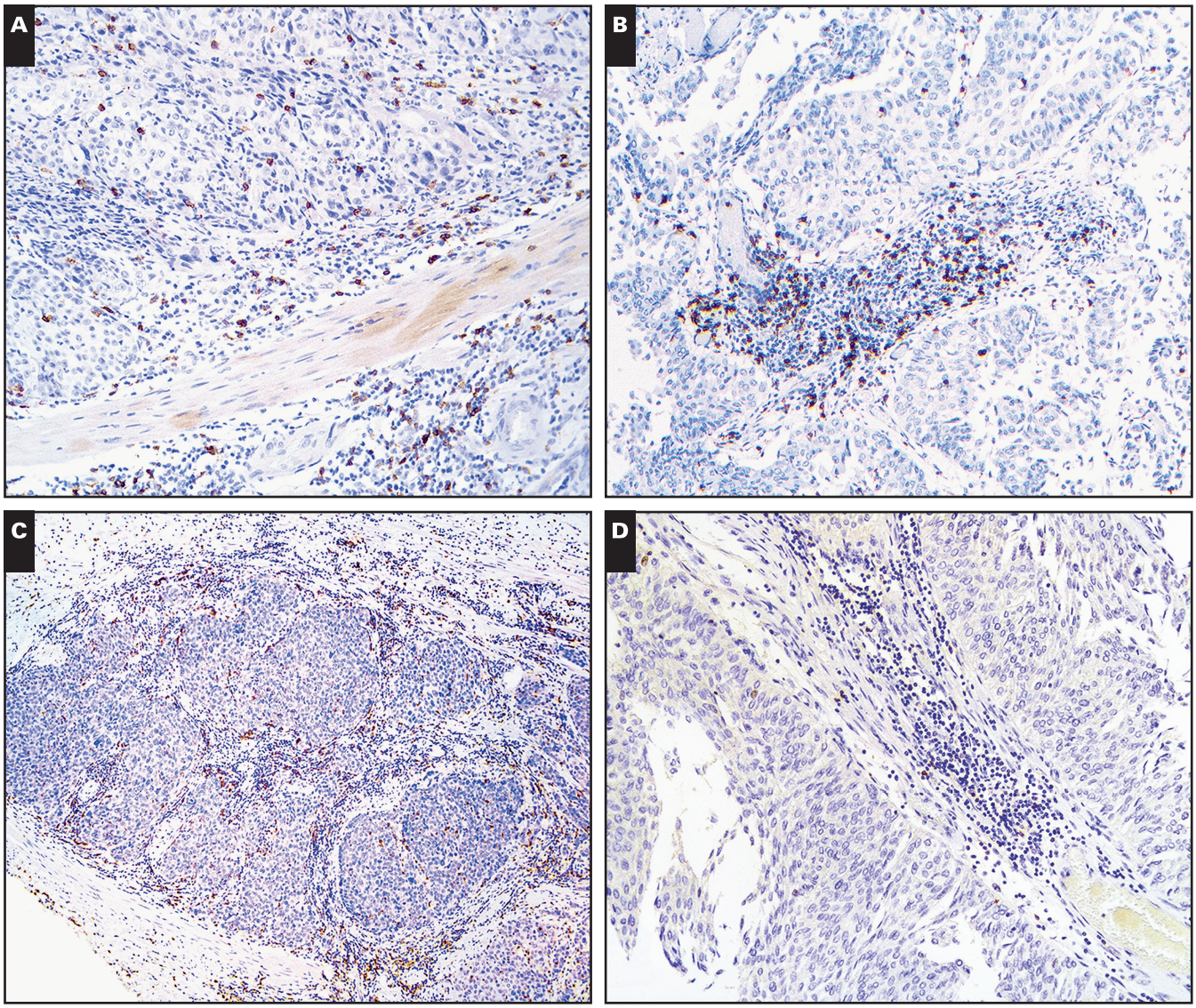

Image 2 CD8 and programmed death 1 receptor (PD-1) tumor-infiltrating lymphocytes in select cases of upper tract urothelial carcinoma: CD8+ intraepithelial and peritumoral lymphocytes (**A**, ×200), CD8+ lymphocytes in papillary cores (**B**, ×200), PD-1+ intraepithelial and peritumoral lymphocytes (**C**, ×100), and PD-1+ lymphocytes in papillary cores (**D**, ×200).

adaptive immune resistance pathway might also be a significant player in such UTUC cases.

Divergent differentiation in UTUC has been shown to be associated with inferior survival.[42-45] Whether this is related to the inherent differences in the TM is unclear. However, the findings in this study, with differences in PD-L1 expression in UTUC with and without squamous differentiation and in the phenotype of peritumoral TILs in PD-L1 positive vs negative cases, underscore this possibility. As the TM is now understood to play a prognostic role in various malignancies,[12,46] it would be worth investigating the relevance of our findings to patient response to PD-1/PD-L1 inhibitors.

Our outcome analysis is notable for a lack of difference in survival or mortality with respect to all of the variables related to the TM. The only significant difference was a shorter disease-free survival in patients with stage T3 tumors. This is in contrast to other studies of PD-L1/PD-1 in UTUC.[32-34] As nearly one-fourth of our cases lacked complete follow-up data, we were only able to include 55 cases in the analysis of disease-free and overall survival (Table 5), which, when broken up by pathologic stage, would include 22 Ta, nine T1, seven T2, and 17 T3 cases. The lack of significant difference in outcome could be explained by the small sample size of our study compared with the other UTUC studies, which included far greater cases (149-423 cases).[32-34]

Limitations of our study include a relatively small sample size, use of a PD-L1 research antibody, and

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Table 5
Clinicopathologic Features and Associated Disease-Free and Overall Survival

	Disease-Free Survival			Overall Survival		
Variable	HR (SE)	95% CI	*P* Value[a]	HR (SE)	95% CI	*P* Value
Tumor size	1.15 (0.08)	1.00-1.31	1.97	1.14 (0.13)	0.91-1.43	1.13
Stage						
T1/T2	3.28 (2.13)	0.92-11.69	.07	5.01 (5.78)	0.52-48.20	.16
T3	4.46 (2.75)	1.33-14.92	**.02**	3.39 (4.15)	0.31-37.42	.32
High-grade histology	0.80 (0.37)	0.32-1.98	.63	1.30 (1.02)	0.28-6.02	.74
Lymph node positive	1.17 (0.40)	0.60-2.29	.65	1.16 (0.63)	0.40-3.38	.79
Surgical margin positive	1.08 (0.66)	0.32-3.61	.90	0.96 (1.01)	0.12-7.53	.97
LVI present or suspicious	0.91 (0.13)	0.69-1.22	.53	0.84 (0.19)	0.53-1.32	.45
Squamous differentiation	1.41 (0.66)	0.56-3.54	.47	0.84 (0.66)	0.18-3.88	.82
CIS present	3.27 (2.51)	0.73-14.68	.12	3.02 (4.28)	0.19-48.42	.43
PD-L1+						
Tumor epithelial	1.73 (0.86)	0.66-4.57	.27	0.97 (0.78)	0.20-4.71	.97
Tumor epithelial with squamous	1.78 (0.88)	0.68-4.70	.24	0.85 (0.68)	0.18-4.10	.84
LH-PCs	1.51 (0.68)	0.63-3.66	.36	0.79 (0.62)	0.17-3.66	.76
LH-PTs	1.25 (0.51)	0.56-2.78	.59	1.12 (0.71)	0.32-3.89	.86
Average CD8+ lymphocytes						
Tumor epithelial	0.96 (0.03)	0.90-1.02	.15	0.97 (0.04)	0.89-1.05	.44
Papillary cores	0.99 (0.01)	0.97-1.01	.39	0.96 (0.02)	0.91-1.01	.09
Peritumoral	1.00 (0.01)	0.97-1.03	.84	0.99 (0.02)	0.94-1.03	.59
Average PD-1+ average lymphocytes						
Tumor epithelial	0.92 (0.06)	0.81-1.05	.23	0.96 (0.07)	0.83-1.11	.60
Papillary cores	0.97 (0.02)	0.93-1.01	.12	0.95 (0.04)	0.87-1.04	.25
Peritumoral	1.00 (0.01)	0.98-1.01	.62	0.98 (0.02)	0.95-1.02	.33

CI, confidence interval; CIS, carcinoma in situ; HR, hazard ratio; LH-PCs, lymphohistiocytes in papillary cores; LH-PTs, peritumoral lymphohistiocytes; LVI, lymphovascular invasion; PD-1, programmed death 1 receptor; PD-L1, programmed death ligand 1; SE, standard error.
[a]Bold value is significant ($P < .05$).

manual counting system of TILs. The PD-L1 E1J2J antibody was used in this study as it was commonly used and extensively validated in our research laboratory prior to the availability of the recent FDA-approved clones. Although there are multiple PD-L1 stains available, recent studies have shown concordance among various clones, including those considered laboratory-developed tests.[47-49] While manual counting has its limitations, it is presently the most commonly used clinical method.

Despite these limitations, our study adds to the limited body of literature on PD-L1 in UTUC. To our knowledge, we are the first to perform a comprehensive analysis of the expression of PD-L1 in various tumor compartments in UTUC along with an evaluation of CD8 and PD-1 TILs. We used whole tissue sections, avoiding the heterogeneity observed in tissue microarrays and small biopsy specimens.[50,51] Ours is also the first study to note a strong association of PD-L1 in UTUC with squamous differentiation. This finding, along with the higher average in PD-1+ peritumoral lymphocytes, emphasizes the unique immunogenicity of such tumors that would need validation in larger cohorts.

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