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Original contribution

Tumor immune microenvironment in non–muscle-invasive urothelial carcinoma of the bladder☆,☆☆,★

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Summary Immunotherapy has gained significance in a variety of tumor types including advanced urothelial carcinoma. Noninvasive urothelial lesions have been treated with intravesical Bacillus-Calmette-Guerin (BCG) for decades. Given treatment failure in a subset of these tumors, ongoing clinical trials investigating the role of checkpoint inhibitors are actively pursued in this group of patients. The present study aims to delineate PD-L1, CD8, and FOXP3 expression in tumor microenvironment in non–muscle-invasive urothelial carcinoma samples obtained via sequential biopsies and to assess its potential role in predicting disease outcome. Cases with >1% and> 5% PD-L1 expression in tumor cells showed lower relative risk (RR) to recur at any subsequent biopsy compared with those with lower PD-L1 expression (RRs, 0.83 [P = .009] and 0.81 [P = .03], respectively). Cases with higher expression of FOXP3 in peritumoral lymphocytes were at lower risk for tumor grade progression at any biopsy (RR, 0.2; P = .02). Tumors with FOXP3/CD8 expression ratio of >1 in intratumoral lymphocytes had lower risk of grade progression (RR, 0.28; P = .04). Although higher number of FOXP3-, CD8-, and PD-L1–positive lymphocytes were encountered after BCG treatment, the findings did not reach statistical significance. In patients without BCG treatment, PD-L1 expression in tumor cells and peritumoral lymphocytes varied across serial biopsies, suggesting the need for additional approaches to assess eligibility for immunotherapy in non–muscle-invasive urothelial carcinoma patients.

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1. Introduction

Urothelial carcinoma of the bladder is the fourth most common malignancy in men in the United States [1]. Most bladder cancer patients present with non–muscle-invasive form of the disease frequently with subsequent recurrences [2,3] that may lead to progression [4]. Intravesical Bacillus-Calmette-Guerin (BCG) therapy, one of the oldest immune therapeutic approaches, remains a standard treatment modality for non–muscle-invasive urothelial carcinoma of the bladder (NMIBC). Despite decreased likelihood of tumor recurrence and progression following BCG therapy [5], lack of response and eventual relapse in some patients remains a significant challenge [6]. Recent resurgence of targeted immunotherapeutics, particularly using antibodies against cytotoxic T-lymphocyte–associated protein 4, programmed cell death protein 1, or programmed death ligand 1 (PD-L1), has positively impacted management of advanced bladder cancer disease [7-10].

PD-L1, a cell surface glycoprotein, acts as an inhibitor of T cells. By its interaction with activated antigen-specific CD8+ T cells, it compromises cytokine production, induces apoptosis in activated CD8+ T cells, and abates their toxicity and therefore plays a crucial role in immune escape [11,12]. Previous studies have shown that a higher number of these intratumoral CD8+ T cells are associated with a better disease-free and overall survival [13]. The interaction of T effector cells and tumor cells is further regulated by many other factors. One of these regulators are regulatory T cells (Tregs), a population of CD4+ T cells, which exhibit forkhead box P3 (FOXP3) and regulate the host immune response by mediating peripheral immune tolerance [14]. The infiltration of tumors by Tregs was shown to be associated with a poor prognosis in several malignancies [15-17]. However, some studies suggest a paradoxical positive prognostic effect of Tregs on various tumor entities including bladder urothelial carcinoma [18]. Furthermore, tumors with a lower ratio of CD8+ T cells to Tregs (CD8+/Treg) were shown to harbor a less favorable outcome [19].

In this study, we sought to investigate several immune markers including PD-L1, FOXP3, and CD8 in NMBIC and to test whether the expression pattern of facets of tumor immune microenvironment has an impact on patient outcome. Furthermore, the impact of BCG treatment on the expression dynamics of such markers and the longitudinal PD-L1 expression overtime in patients without BCG treatment was also evaluated.

2. Materials and methods

2.1. Patient cohort and tissue microarray construction

The study was approved by the George Washington University Institutional Review Board and was deemed exempt from informed consent.

Three tissue microarrays were constructed from 143 transurethral resection of bladder tumor (TURBT) specimens from 61 patients treated at George Washington University between 1998 and 2008. In every patient, the initial TURBT specimen at time of de novo diagnosis and all available subsequent TURBT samples during follow-up were included. Tumor diagnosis included carcinoma in situ (CIS), low- and high-grade noninvasive papillary urothelial carcinoma (LGTa and HGTa, respectively), and invasive urothelial carcinoma (pT1 and higher). All sections were retrieved and reviewed by a urologic pathologist for confirmation of the original diagnosis, grade, and stage according to the World Health Organization 2016 classification [20] and the eighth edition of the AJCC Cancer staging manual [21].

Representative paired formalin-fixed, paraffin-embedded tumor and, whenever available, nontumor samples were spotted 3 to 6 times, resulting in a total of 461 tissue microarray (TMA) spots. These spots included 48 cores of nontumor tissue, 21 cores of CIS, 116 cores of low-grade noninvasive papillary urothelial carcinoma, 168 cores of high-grade noninvasive papillary urothelial carcinoma, and 108 cores of non–muscle-invasive urothelial carcinomas.

Follow-up data on recurrence, grade or stage progression, and treatment were obtained from medical records, with a follow-up period ranging from 2 to 275 months (mean, 43 months).

2.2. Immunohistochemistry analysis

Three genitourinary pathologists (G.G., D.T., M.A.M.R.) evaluated all tumor spots and corresponding benign tissue.

Antibodies were acquired from commercial sources as implemented as follows: FOXP3 (eBioscience (eBioscience is now part of Thermo Fisher Scientific (Waltham, MA, USA)); 14-4777) 1:250 diluted in antibody dilution buffer with an overnight incubation at 4°C, CD8 (Thermo Fisher Scientific (Waltham, MA, USA) RB-9009-P0) 1:900 diluted in antibody dilution buffer with a 45-minute incubation at room temperature, PD-L1 (Cell Signaling (Danvers, MA, USA) clone E1L3N, no. 13684) 1:100 diluted in antibody dilution buffer with a 45-minute incubation at room temperature, and Ki-67 (Invitrogen/Thermo Fisher (part of Thermo Fisher Scientific (Waltham, MA, USA)); 33-4711) diluted 1:900 in antibody dilution buffer.

Detection of immunolabeling was performed using anti-mouse or antirabbit horseradish peroxidase–conjugated secondary antibodies and developed using 3-3-*O*-diaminobenzidine or 3-amino-9-ethylcarbazole chromogen and counterstained with hematoxylin. Appropriate negative and positive controls were used.

2.3. Scoring system

For all markers, the maximum value of the marker across TMA spots in a given case was assigned as the expression score and used for further analysis. In paired "nontumor" tissue, measurements were assessed in benign urothelial cells, and intraepithelial and periepithelial lymphocytes.

PD-L1 expression was separately scored in tumor cells and in peritumoral lymphocytes as the percentage of positive cells.

Table 1 Clinical and outcome features of 61 patients with non–muscle-invasive bladder urothelial carcinoma

Features	Values
Age (y), mean (range)	68 (47–89)
Sex, n (%)	
Male	41 (67)
Female	20 (33)
Tumor recurrence [a], n (%)	
Yes	52 (85)
No	9 (15)
Tumor grade progression [a], n (%)	
Yes	5 (8)
No	56 (92)
Tumor stage progression [a], n (%)	
Yes	6 (10)
No	55 (90)
Overall mortality	
Alive	49 (80)
Dead (all causes)	6 (10)
NA	6 (10)
Follow-up (mo), mean (range)	43 (2, 275)

Abbreviation: NA, not available.
[a] At any time during follow-up.

PD-L1 expression scores were then categorized as low versus high using 2 different cutoff points: 1% and 5%, in both tumor cells and peritumoral lymphocytes.

FOXP3, CD8, and Ki-67 were scored as the number positive cells per high-power field. Intratumoral and peritumoral lymphocytes were separately scored. The proliferative fraction of CD8+ T cells, indicated by dual CD8/Ki-67 positivity, was similarly assessed. Using the overall median scores per marker as the cutoff point, FOXP3, CD8, and dual CD8/Ki-67 expressions were categorized as low versus high expression.

2.4. Data analysis

Data analysis was carried out using paired events. For any given case, the paired event consisted of 2 consecutive TURBT diagnoses during follow-up. Thus, a case could have one or more paired events. For each marker, the considered outcomes included 1 of 6 possibilities: (i) tumor recurrence at next biopsy diagnosis (tumor reappeared showing a similar or lower-grade/stage lesion), (ii) tumor recurrence at any subsequent biopsy diagnosis (tumor recurred at least once during follow-up), (iii) tumor grade progression at next biopsy diagnosis (tumor reappeared showing a higher-grade lesion), (iv) tumor grade progression at any subsequent biopsy diagnosis (tumor showed grade progression at least once during follow-up), (v) tumor stage progression at next biopsy diagnosis (tumor reappeared showing a higher-stage lesion), and (vi) tumor stage progression at any subsequent biopsy diagnosis (tumor showed stage progression at least once during follow-up).

For the association analysis, we used the Kruskal-Wallis rank sum test. The Wilcoxon rank sum test was used as a post hoc test for assessing pairwise associations. For outcome analysis, relative risks (RRs) were estimated by unconditional maximum likelihood estimation with 95% confidence intervals (CIs)

Table 2 Marker expression per histologic category

	Nontumor [a] mean median	CIS mean median	LGTa mean median	HGTa mean median	Invasive mean median	P [b]
PD-L1 expression						
Tumor cells (%)	0.7 0	0.9 0	5 0	3 0	3.6 0	**.00073**
Peritumoral lymphocytes (%)	1.8 0	4.2 0	2.8 0	10.4 1.0	19.4 10.0	**<.0001**
FOXP3 expression						
Intratumoral lymphocytes	17 17	0.7 1.0	1.8 0	2.2 1.0	3.7 2.0	**.00076**
Peritumoral lymphocytes	43.5 13.5	35.3 28.0	24.4 5.0	32.2 8.0	32.7 17.0	**.0028**
CD8 expression						
Intratumoral lymphocytes	20 20	4.6 2.0	12.0 6.0	5.9 1.0	9.0 1.0	**<.0001**
Peritumoral lymphocytes	33.1 23	27.3 15.0	22.7 6.0	21.9 5.0	26.8 8.0	.062
Dual CD8/Ki-67 expression						
Intratumoral lymphocytes	0 0	0.1 0	0.1 0	0.5 0	0.3 0	.48
Peritumoral lymphocytes	0 0	0.1 0	0.1 0	0.1 0	0.1 0	.58

Abbreviations: HGTa, high-grade noninvasive papillary urothelial carcinoma; LGTa, low-grade noninvasive papillary urothelial carcinoma.
[a] In nontumor tissues, measurements were assessed in paired urothelial cells, and intraepithelial and periepithelial lymphocytes.
[b] From the Kruskal-Wallis rank sum test.

calculated using normal approximation (Wald). *P* values were calculated using the Barnard unconditional exact test. For all markers, expression before and after BCG treatment was compared using the Wilcoxon signed rank test with continuity correction. For PD-L1 expression, variability across sequential biopsies in patients without BCG therapy was also assessed.

Statistical significance required 2-tailed $P < .05$. For pairwise comparisons, *P* values were adjusted using the Bonferroni method. Data were analyzed and plots were generated using R version 3.5.3 (2019-03-11) from the R Foundation for Statistical Computing (Vienna, Austria). All the data, code, and results are available as supplementary materials in a GitHub repository at https://github.com/alcideschaux/PFCK-NMIBC/.

3. Results

3.1. Clinicopathological features

Clinicopathological parameters are shown in Table 1. Sixty-one patients were included in the study. Twenty patients ware female, and 41 were male. The mean age was 68 years (range from 47 to 89 years). Patients were followed up for a mean of 43 month (range from 2 to 275 months). On follow-up, 52 patients experienced tumor recurrence, 5 patients developed tumor grade progression, and 6 patients showed tumor stage progression. The overall mortality rate was 10%.

3.2. Association of maker expression with histologic categories

Mean and median PD-L1, FOXP3, CD8, and dual CD8/Ki-67 expression by histologic category are shown in Table 2. Representative staining is depicted in Fig. 1. We found significant variability in PD-L1 expression among cases with many tumors lacking expression PD-L1 expression in tumor cells as well as tumor-infiltrating lymphocytes (median values of 0% and 1%, respectively). Across histologic categories, the highest mean tumor cell expression of PD-L1 was found in LGTa (mean of 5.0%, $P = .0007$), with significant differences between invasive carcinomas and LGTa (3.6 versus 5 mean percentages, $P = .002$) and between invasive carcinomas and HGTa (3.6 versus 2.9 mean percentages, $P = .03$). In peritumoral lymphocytes, the highest PD-L1 expression was found

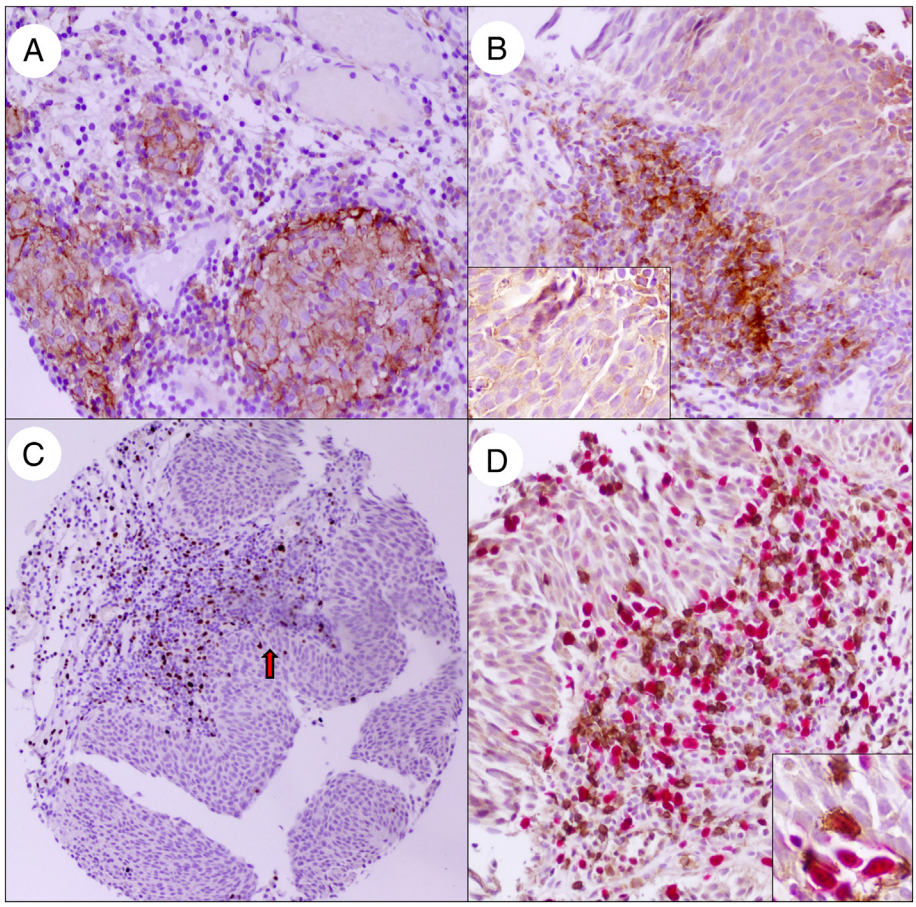

Fig. 1 PD-L1 immunohistochemical expression. A, Strong membranous and cytoplasmic PD-L1 positivity in granulomas (×200). B, High-grade noninvasive urothelial carcinoma with cytoplasmic and membranous PD-L1 expression. PD-L1 positivity is also present in tumor-related mononuclear inflammatory cells (×200) and tumor cells (inset, ×400). C, Noninvasive high-grade urothelial carcinoma. FOXP3-positive lymphocytes are numerous in the stroma and rare in the tumor (arrow; ×100). D, CD8 (brown)–Ki-67 (red) dual stain demonstrates numerous lymphocytes, predominantly in stroma adjacent to tumor (×200). Rare CD8-positive cells are also Ki-67 positive, indicating proliferation (inset; ×400).

Table 3 RRs of outcomes by biomarker expression

	Recurrence at next biopsy		Recurrence at any biopsy		Stage progression at next biopsy		Stage progression at any biopsy		Grade progression at next biopsy		Grade progression at any biopsy	
	RR (95% CI)	*P*	RR (95% CI)	*P*	RR (95% CI)	*P*	RR (95% CI)	*P*	RR (95% CI)	*P*	RR (95% CI)	*P*
PD-L1 expression (>1%)												
Tumor cells	0.94 (0.65–1.35)	.74	0.83 (0.68–1)	**.009**	0.51 (0.06–4.07)	.59	1.88 (0.74–4.75)	.20	0.49 (0.06–3.89)	.59	0.94 (0.28–3.2)	.94
Peritumoral lymphocytes	0.9 (0.63–1.29)	.61	0.98 (0.85–1.12)	.79	0.27 (0.03–2.47)	.26	2.53 (0.53–11.95)	.24	0.81 (0.12–5.49)	.85	0.42 (0.11–1.59)	.20
PD-L1 expression (>5%)												
Tumor cells	0.96 (0.63–1.45)	.89	0.81 (0.63–1.05)	**.03**	0.8 (0.1–6.25)	.89	1.8 (0.65–5.02)	.31	NA	.25	0.98 (0.24–4.1)	1
Peritumoral lymphocytes	0.84 (0.58–1.23)	.38	1.01 (0.88–1.16)	.97	0.44 (0.05–4.03)	.61	4 (0.85–18.9)	*.06*	0.43 (0.05–3.99)	.59	0.38 (0.08–1.75)	.22
High FOXP3 expression												
Intratumoral lymphocytes	0.88 (0.65–1.18)	.39	0.97 (0.88–1.08)	.65	0.34 (0.04–2.94)	.36	1.4 (0.53–3.74)	.61	0.94 (0.16–5.36)	.97	0.6 (0.16–2.21)	.57
Peritumoral lymphocytes	0.9 (0.68–1.2)	.53	1.02 (0.92–1.12)	.82	0.39 (0.08–1.94)	.25	0.7 (0.28–1.72)	.53	0.2 (0.02–1.69)	.12	0.2 (0.05–0.88)	**.02**
High CD8 expression												
Intratumoral lymphocytes	1.11 (0.83–1.5)	.53	1.08 (0.97–1.2)	.16	0.63 (0.11–3.58)	.62	0.75 (0.28–2.02)	.68	NA	.04	2.3 (0.64–8.56)	.24
Peritumoral lymphocytes	1 (0.75–1.33)	1	0.98 (0.89–1.08)	.82	0.42 (0.09–2.09)	.30	0.89 (0.37–2.15)	.87	0.53 (0.1–2.76)	.53	0.71 (0.24–2.13)	.59
High dual CD8/Ki-67 expression												
Intratumoral lymphocytes	0.89 (0.63–1.24)	.50	0.93 (0.81–1.06)	.24	0.52 (0.06–4.43)	.61	0.64 (0.19–2.13)	.48	NA	.23	0.26 (0.03–1.96)	.17
Peritumoral lymphocytes	1.05 (0.74–1.47)	.85	1.05 (0.96–1.15)	.48	0.58 (0.07–4.61)	.74	0.44 (0.11–1.82)	.26	0.73 (0.09–5.98)	.84	1.1 (0.32–3.81)	.91
FOXP3/CD8 ratio >1												
Intratumoral lymphocytes	0.94 (0.7–1.26)	.74	0.95 (0.86–1.06)	.47	2.91 (0.34–25.05)	.36	3.85 (0.91–16.37)	.05	NA	.01	0.28 (0.08–1.01)	**.04**
Peritumoral lymphocytes	0.99 (0.74–1.32)	.99	1.03 (0.93–1.14)	.63	0.63 (0.15–2.7)	.63	0.78 (0.32–1.89)	.63	0.44 (0.08–2.31)	.37	0.44 (0.14–1.38)	.17

Abbreviation: NA, not available (ie, RR cannot be computed because of low cell counts); RR, risk ratio.

in invasive tumors (mean of 19.4%, $P < .0001$), with significant differences between invasive carcinomas and nontumor tissue (means of 19.4% versus 1.8%, $P = .0002$), CIS (means of 19.4% versus 4.2%, $P = .004$), LGTa (means of 19.4% versus 2.8%, $P < .0001$), and HGTa (means of 19.4% versus 10.4%, $P = .002$).

Highest FOXP3 expression was found in intraepithelial (mean of 17 counts) and periepithelial lymphocytes (mean of 44 counts) in nontumor tissue ($P = .0008$ and $P = .003$, respectively), with significant differences between invasive tumors and LGTa (3.7 versus 1.8 mean counts, $P = .002$) for FOXP3+ intratumoral lymphocytes and between LGTa and CIS (24.4 versus 35.3 mean counts, $P = .005$) for FOXP3+ peritumoral lymphocytes.

Highest counts of CD8+ intratumoral lymphocytes were found in nontumor tissues (mean of 20 counts, $P < .0001$), with significant differences between LGTa and HGTa (means of 12 versus 5.9, $P < .0001$) and between LGTa and invasive carcinomas (means of means of 12 versus 9, $P = .0001$). The differences in CD8 expression of peritumoral lymphocytes across histologic categories did not reach statistical significance ($P = .06$), although a trend for higher counts of CD8+ cells in nontumor urothelium was observed. The proliferating fractions of CD8+ lymphocytes (both intratumoral and peritumoral) were low across all histologic categories. Full values of the distribution, association tests, and post hoc tests can be found at the GitHub repository.

3.3. Association of marker expression with outcome

Outcome analysis of markers is displayed in Table 3. Using >1% as cutoff, cases with high PD-L1 expression in tumor cells were at lower risk to develop tumor recurrence at any biopsy compared with those with lower PD-L1 expression (RR,

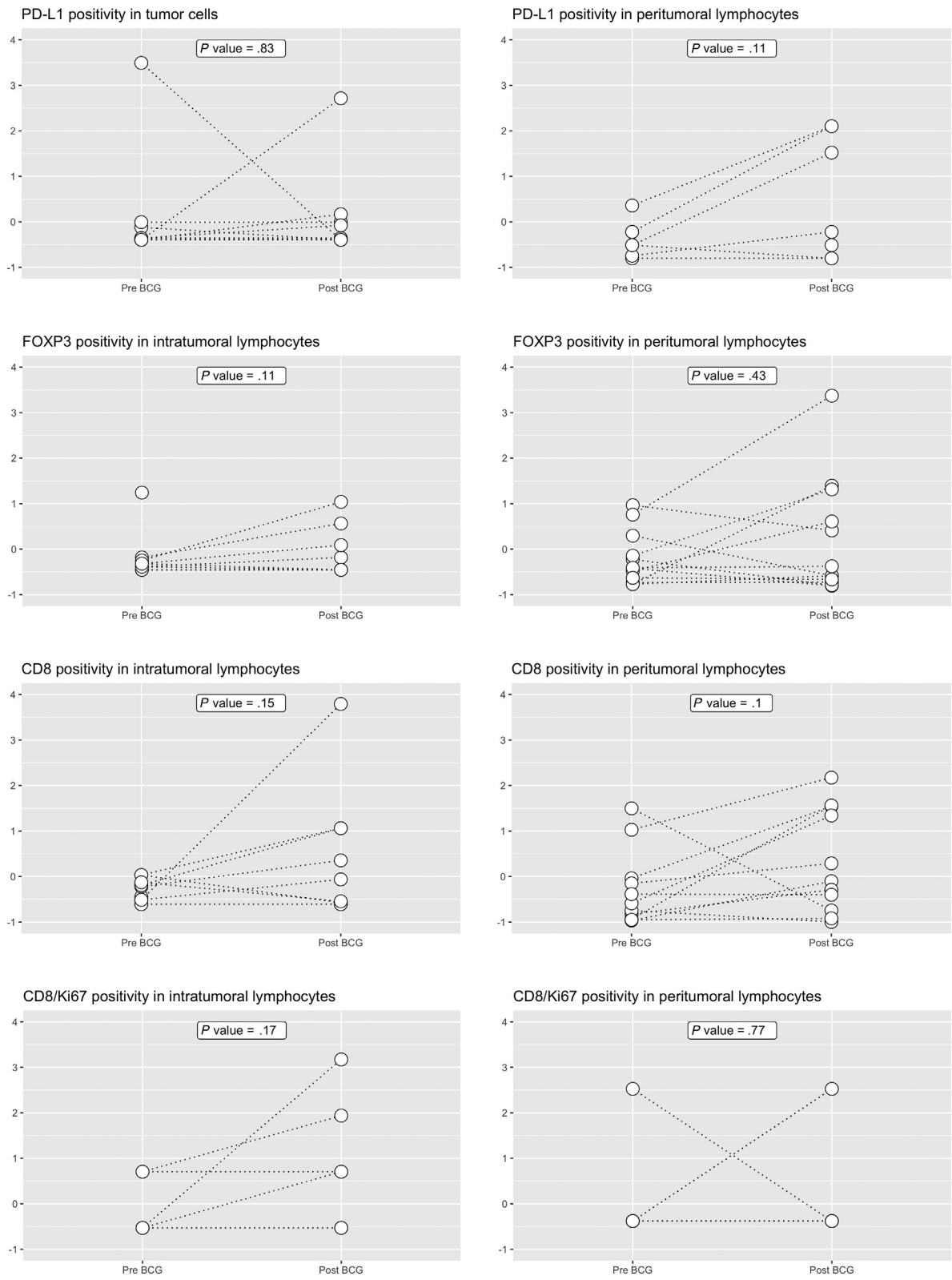

Fig. 2 PD-L1, FOXP3, and dual CD8/Ki-67 maximum expression before and after BCG treatment in a subset of 12 non–muscle-invasive bladder cancer patients. Expression levels are scaled to allow for comparison across markers. Value of 0 at the *y*-axis represents the mean with each unit representing an SD. White circles represent the scaled value for each patient. Dashed lines represent the trend before and after BCG treatment. *P* values were calculated using the Wilcoxon signed rank test with continuity correction.

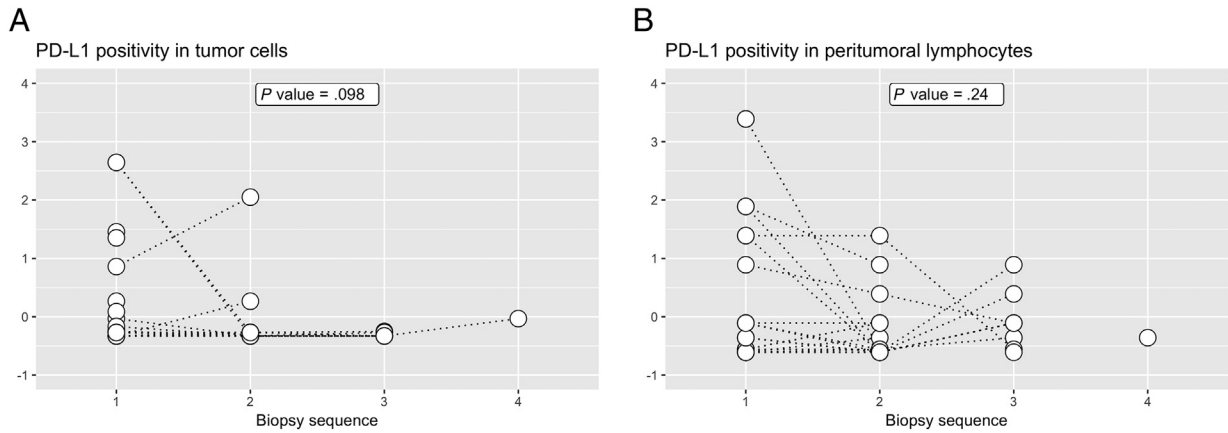

Fig. 3 PD-L1 expression in tumors cells and peritumoral lymphocytes in cases without BCG treatment. Inconsistency in PD-L1 expression in tumor cell (A) and peritumoral lymphocytes (B) is noted in individual patients. Expression levels are scaled to allow for comparison across markers. Value of 0 at the *y*-axis represents the mean with each unit representing an SD. White circles represent the scaled value for each patient. Dashed lines represent the trend before and after BCG treatment. *P* values were calculated using the Wilcoxon signed rank test with continuity correction.

0.83; $P = .009$). Similarly, tumor cells with >5% of PD-L1 positivity showed less risk for tumor recurrence at any biopsy (RR, 0.81; $P = .03$).

Regarding FOXP3 expression, cases with higher density of FOXP3+ peritumoral lymphocytes showed less risk for tumor grade progression at any biopsy (RR, 0.2; $P = .02$). Neither CD8 expression nor dual CD8/Ki-67 expression showed any relevant effect on outcome. Finally, tumors with intratumoral lymphocytes FOXP3/CD8 ratio of >1 showed less risk of tumor grade progression at any biopsy diagnosis (RR, 0.28; $P = .04$).

3.4. Marker expression in relationship to BCG treatment

To examine whether BCG treatment had an effect on the immune markers, we investigated PD-L1, FOXP3, and dual CD8/Ki-67 in tumor specimens from patients before and after BCG treatment. In our cohort, 19 patients received BCG therapy. Of these, 12 patients had pre- and post-BCG scoring data available. None of the examined markers showed statistically significant change after BCG treatment (Fig. 2). Trends for increased number of positive lymphocytes were noted for PD-L1 (peritumoral), FOXP3 (intratumoral), and CD8 (intratumoral).

3.5. PD-L1 expression dynamics in cases without BCG treatment

We investigated PD-L1 expression in tumor cells and peritumoral lymphocytes in sequential biopsies of patient who did not receive BCG treatment. Overall, no statistically significant differences in PD-L1 expression over time were observed (Fig. 3). However, inconsistency in PD-L1 expression in tumor cell as well as in peritumoral lymphocytes was noted in individual patients (see Fig. 3).

4. Discussion

Immune microenvironment has recently been a field of active investigations in bladder cancer. For advanced disease, checkpoint inhibitors have been approved as a second-line therapy in platinum-refractory cases and as first-line treatment in cisplatin-ineligible patients [22]. Eligibility for checkpoint inhibitor treatment has been linked to immunohistochemical PD-L1 positivity in tumor cells or tumor-infiltrating lymphocytes. Cutoff points for PD-L1 positivity and antibody clones vary across studies [7,23-27]. In NMIBC, intravesical BCG treatment remains the criterion standard. Given treatment failure in a subset of these tumors, ongoing clinical trials investigating the role of checkpoint inhibitors are actively pursued [22]. Studies detailing immune microenvironment in NMIBC are valuable.

Analysis of our sequential TURBT specimens of NMIBC patients suggests a significant variability in PD-L1 expression with many tumors lacking expression. Cases with >1% and> 5% positive tumor cells were less likely to recur at any following biopsy diagnosis. Previous studies assessing possible prognostic role of PD-L1 expression in bladder carcinoma have shown inconsistent results [28-30]. In their study of NMIBC and MIBC patients, Huang et al [28] found PD-L1 mRNA expression to be higher in advanced-stage tumors. Higher PD-L1 levels were also associated with reduced patient survival. In line with their study, we found higher peritumoral lymphocyte PD-L1 expression in ≥pT1 tumors compared with noninvasive lesions. Higher PD-L1 expression in peritumoral lymphocytes showed a trend of a greater likelihood of tumor stage progression at any biopsy. These findings are in contrast to studies focusing on MIBC. Eckstein et al [31] showed that MIBC tumors with >1% PD-L1–positive immune cells had a better overall survival.

In patients without BCG treatment, we found PD-L1 expression to vary between biopsies from the same patient

suggesting the need for additional approaches to assess eligibility for immunotherapy in NMIBC patients besides PD-L1 positivity.

FOXP3, which is widely used as a marker for Tregs, was previously shown to be associated with decreased survival in bladder cancer [15]. In their study of 115 primary NMIBCs, Murai et al [32] revealed that percentage of FOXP3+ T cells was an independent predictor of tumor recurrence. In our study, we observed no association with tumor recurrence. However, decreased risks for grade progression at any time were associated with higher FOXP3 expression in peritumoral lymphocytes. The study by Murai et al [32] found no change in total number of FOXP3+ T cells after BCG treatment. Although in our study we found a trend for increased for FOPX3 positivity in intratumoral lymphocytes, the findings did not reach statistical significance. Additional studies in larger cohorts are needed to validate these findings.

A higher number of intratumoral CD8+ T cells have been associated with a better disease-free and overall survival in MIBC [13]. In contrast, studying PUNLMP and LGTa tumors, Krpina et al [33] showed that the higher number of CD8+ cells was associated with disease recurrence. We did not find any significant associations between CD8+ lymphocytes expression and outcome.

The ratio of FOXP3/CD8 expression has been assessed in relationship to outcome in bladder cancer [19]. Le Goux et al [34] found an increased likelihood of recurrence in NMIBC tumors showing high FOXP3/CD8 mRNA levels. However, we found decreased risks for grade progression at any future biopsy in cases showing intratumoral lymphocytes with a FOXP3/CD8 ratio >1.

Our study has some limitations, such as the retrospective design and the relatively small number of patients with BCG therapy. Furthermore, TMA assays are less likely to capture the topographic heterogeneity of markers expression within a given tumor. On the other hand, TMA design allows for consistency and standardization of immunohistochemical variables [35]. The detailed multimarker assessment in both tumor and microenvironment compartments and the use of a sequential single institution cohort are some of the strengths of our study.

In summary, we found higher expression of PD-L1 in tumor cells to be associated with a lower risk for recurrence. Although not statistical significant, we found an increased number of FOXP3+, CD8+, and PD-L1+ T cells after BCG treatment. In patients without BCG treatment, PD-L1 expression varied among biopsies, potentially indicating the need for additional approaches to assess eligibility for immunotherapy in NMIBC patients.

CRediT authorship contribution statement

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