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ORIGINAL RESEARCH

The ratio of CD8 to Treg tumor-infiltrating lymphocytes is associated with response to cisplatin-based neoadjuvant chemotherapy in patients with muscle invasive urothelial carcinoma of the bladder

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ABSTRACT

Introduction: Randomized controlled trials of platinum-based neoadjuvant chemotherapy (NAC) for bladder cancer have shown that patients who achieve a pathologic response to NAC exhibit 5 y survival rates of approximately 80–90% while NAC resistant (NR) cases exhibit 5 y survival rates of approximately 30–40%. These findings highlight the need to predict who will benefit from conventional NAC and the need for plausible alternatives. **Methods**: The pre-treatment biopsy tissues from a cohort of 41 patients with muscle invasive bladder who were treated with NAC were incorporated in tissue microarray and immunohistochemistry for PD-L1, CD8, and FOXP3 was performed. Percentage of PD-L1 positive tumor cells was measured. Tumor-infiltrating lymphocytes (TIL) densities, along with CD8 and Treg-specific TILs, were measured. **Results**: TIL density was strongly correlated with tumor PD-L1 expression, consistent with the mechanism of adaptive immune resistance in bladder cancer. Tumor cell PD-L1 expression was not a significant predictor of response. Neither was the CD8 nor Treg TIL density associated with response. Intriguingly though, the ratio of CD8 to Treg TIL densities was strongly associated with response ($p = 0.0003$), supporting the hypothesis that the immune system plays a role in the response of bladder cancer to chemotherapy. **Discussion**: To our knowledge, this is the first report in bladder cancer showing that the CD8 to Treg TIL density in the pre-treatment tissues is predictive for conventional NAC response. These findings warrant further investigations to both better characterize this association in larger cohorts and begin to elucidate the underlying mechanism(s) of this phenomenon.

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Introduction

Urothelial carcinoma of the bladder is the fourth most common cancer seen in men.[1] It represents also the second most common malignancy of the genitourinary tract, and the second most common cause of death among genitourinary tumors.[2] Radical cystectomy (RC) with urinary reconstruction is considered to be the gold standard treatment for muscle-invasive urothelial carcinoma (MIBC).[3] Randomized controlled trials and subsequent meta-analyses comparing cisplatin-based neoadjuvant chemotherapy (cNAC) following by RC versus RC alone has demonstrated a small but significant survival benefit associated with platinum-based combination chemotherapy.[4-7] Careful review of the randomized controlled trials of platinum-based combination NAC highlights an important phenomenon: patients who achieve a pathologic response to NAC (defined as the absence of muscle invasive disease at RC following cNAC) have a 5-y survival rate of approximately 80–90% while those with NR MIBC have a 5-y survival rate of approximately 30–40%; this is a robust difference and is notably different than the 50% 5-y survival for patients with MIBC treated by cystectomy alone. The overall response rate to cNAC is 40–50%; therefore, a little over half of patients with MIBC would be amenable to an alternative therapeutic intervention prior to RC if one were available and efficacious.[8]

Programmed death ligand 1 (PD-L1), also known as B7-H1 or CD274,[9] is a cell surface glycoprotein which inhibits T cell-mediated antitumor immunity through interaction with PD-1, by attenuating T cell activation and effector function.[10] Although PD-L1 message is widely expressed, expression at the protein level is limited to cells of the macrophage lineage and inflamed tissues, where it likely prevents excessive tissue damage at sites of infection/inflammation. Cancer cell expression of PD-L1 occurs in many malignancies, including but not limited to bladder cancer, melanoma, renal cell carcinoma, lung, ovary, breast, endometrium, glioblastoma, gastric, gastrointestinal, esophageal, and hepatocellular carcinoma.[11,12] Furthermore, PD-L1 has been shown to be expressed in about 15–35%

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of urothelial carcinomas of post-cystectomy patients, and increased expression is generally associated with worse cancer-specific outcomes.[13,14] Recently, in patients with metastatic urothelial carcinoma, inhibition of the immune checkpoint inhibitor PDL-1 using anti-PD-L1 antibody MPDL3280A was associated with a response rate of approximately 30% in unselected patients, and a favorable tolerability profile.[7]

The engagement of the host immune system by an evolving malignancy is complex, involving a myriad of cell types and cell signaling phenomena. Most antitumor effects of the host immune system can be broadly ascribed to effector CD8 (Killer) T cells, although other cell types certainly play a role. Recent evidence has shown that CD8 T-cells play a role in MIBC; in that tumors with increased CD8 lymphocyte infiltrate exhibit favorable post-cystectomy CSS.[15] Opposing the antitumor activity of CD8 T cells is a population of $CD4^+$ T cells that express the forkhead box P3 (FOXP3) transcription factor, and which have a regulatory activity on the host immune response (known as Tregs).[16] Tregs play a crucial role in maintaining immune homeostasis and mediating peripheral tolerance, accordingly it has been shown that mice lacking FoxP3 develop an autoimmune phenotype.[17] Infiltration by regulatory T cells has been reported in a variety of malignancies including MIBC, lung cancer, breast cancer, and others in which their presence tends to portend a poor prognosis.[18-20] Further, lower effector CD8 T cells to Treg (CD8/Treg) ratios correlate with a less favorable outcome in multiple tumor types.[21]

The objective of this study was to evaluate PD-L1 expression in the chemotherapy naive biopsy specimens of MIBC and to test the hypothesis that aspects of the lymphocytic component of the tumor immunologic microenvironment correlates with the response to cNAC.

Results

Cohort characteristics

Transurethral resection of bladder tumor (TURBT) specimens from a cohort of 67 patients with chemotherapy naïve MIBC were incorporated into tissue microarrays and examined in this study (Table 1 and Table S1). For 41 of these patients, there was adequate chemotherapy dosing information to establish that these patients were able to tolerate a therapeutic dose of platinum-based induction chemotherapy; thereby allowing for the adequate assessment of chemotherapy response in the resultant cystectomy specimen. We previously reported on patients from this cohort in two related studies aiming to identify clinical and molecular factors predictive of response to cNAC.[22,23] As compared to the cohort as a whole, there was a small increase in the proportion of Caucasians present in the cNAC cohort subset, which was statistically significant (Fisher's Exact test $p = 0.041$). Not unexpectedly, there was an enrichment for pathologic tumor stage 0 (pT0) in the cNAC cohort subset, which approached statistical significance Fisher's Exact test $p = 0.10$. No significant biases were noted in the cNAC cohort subset with respect to age, sex, clinical stage, and pathologic node stage.

Table 1. Patients demographics of the MIBC cohort examined and the cNAC subset. The cohort demographics are shown above, with the column-wise percentages shown in gray text. No significant biases were observed in the cNAC subset with respect to age, sex, clinical tumor stage, and pathologic node stage. A small increase in Caucasians and pT0 (marked by *) was observed in the cNAC subset, Fisher's Exact test p value 0.04 and 0.10, respectively.

		MIBC TURBTs n = 67		cNAC Cohort n = 41	
Age					
	Median [min, max]	62 [45, 82]		64 [45, 82]	
Race				*	
	Cauc.	52	*78%*	36	*88%*
	Afr. Amer.	9	*13%*	3	*7%*
	Other	6	*9%*	2	*5%*
Sex					
	Male	55	*82%*	33	*80%*
	Female	12	*18%*	8	*20%*
Clinical T Stage					
	<= cT2	33	*49%*	19	*46%*
	cT3	19	*28%*	15	*37%*
	cT4	12	*18%*	7	*17%*
Pathologic T Stage				*	
	pT0	12	*18%*	10	*24%*
	pTa/Tis/T1	13	*19%*	6	*15%*
	pT2	4	*63%*	2	*61%*
	pT3	31		17	
	pT4	7		6	
Pathologic N Stage					
	Negative	36	*54%*	23	*56%*
	Positive	17	*25%*	11	*27%*

MIBC PD-L1 expression correlates with tumor-infiltrating lymphocyte (TIL) density

The degree of PD-L1 tumor expression in MIBC was strongly correlated with overall TIL density (Fig. 1A (Goodman–Kruskal γ=0.68 $p < 0.0001$). This association was also present when the TIL density was subtyped based on CD8 staining (Goodman–Kruskal $\gamma = 0.57$ $p = 0.002$) or FOXP3 staining (Goodman–Kruskal $\gamma = 0.73$ $p < 0.0001$). These findings are consistent with prior data from cystectomy specimens in which a strong association of tumor PD-L1 status and the degree of CD8 TILs was also observed (Fishers exact test $p = 0.01$).[15] Given the relationship of the CD8/Treg ratio with outcome in several tumor types, we also tested whether this ratio correlated with PD-L1 expression. However, as shown in Fig. 1B, the CD8/Treg ratio was not statistically associated with expression of PD-L1 in this MIBC cohort (Fig. 1B, Goodman–Kruskal $p = 0.29$). We focused on tumor PD-L1 staining, as we previously found it to be more predictive of response to anti-PD-1 checkpoint blockade than PD-L1 expression on infiltrating immune cells.[24] Occasional staining in myeloid cells and stromal lymphocytes was noted, but in these MIBC samples PD-L1 TIL staining was scant.

CD8/Treg ratio (but not tumor cell PD-L1 expression) correlates with cNAC response

Given the previously described relationship between PD-L1 expression and outcome in urothelial carcinoma,[25] we tested whether expression correlated with response to cNAC. This was not the case, as shown in Fig. 2 pre-treatment tumor

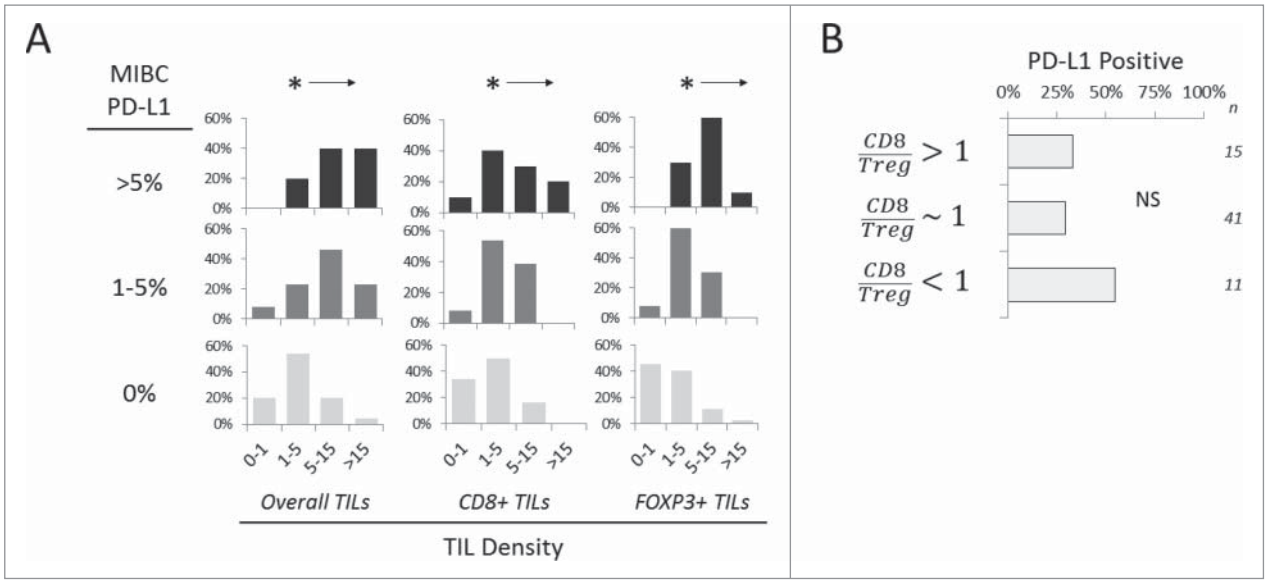

Figure 1. Spectrum of tumor-infiltrating lymphocytes characterized by MIBC PD-L1 staining. (A) Normalized histograms are shown with the TIL densities (TIL counts per 100 tumors cells) binned into the designated intervals on the *x*-axes and the sample proportions shown on the *y*-axes. In each case (overall, CD8, and FOXP3$^+$ TILs) a significant (*Goodman–Kruskal $p < 0.01$) increase in the amount of TILs is observed with increased MIBC PD-L1 staining. (B) The percentage of cases with MIBC PD-L1 positivity (defined as > 0%) is shown on the *x*-axes and the per sample CD8/Treg ratio on the *y*-axes. No statistically significant (NS) association to MIBC PD-L1 expression status was observed.

PD-L1 expression was not statistically different in cNAC responders as compared to resistant cases.

We next tested whether pretreatment total TIL density, as well as CD8 and FoxP3 density were associated with cNAC response. Interestingly, none of these three parameters showed a clear association with response (Fig. 3A, Goodman–Kruskal $p > 0.05$). Finally, we tested whether the ratio of CD8/Treg cells, potentially a more integrative correlate of an adaptive immune response, was associated with the response to cNAC. Surprisingly, and in contrast to what was observed with PD-L1 expression, the ratio of CD8 to FOXP3 TIL densities was significantly associated with cNAC response (Fig. 3B, Goodman–Kruskal $\gamma = 0.66$ $p = 0.0003$). Tumors in which the CD8/FOXP3+ ratio was > 1 exhibited a cNAC response rate of 60% (n = 10). Tumors in which the CD8 TIL density was approximately equivalent to the FOXP3$^+$ TIL density exhibited a NAC response rate of 42% (n = 24). And intriguingly, tumors in which the CD8/FoxP3 ratio was < 1, showed no responses (n = 7).

Discussion

This study quantified tumor cell PD-L1 expression as well as CD8 and Treg infiltration in TUR bladder cancer specimens

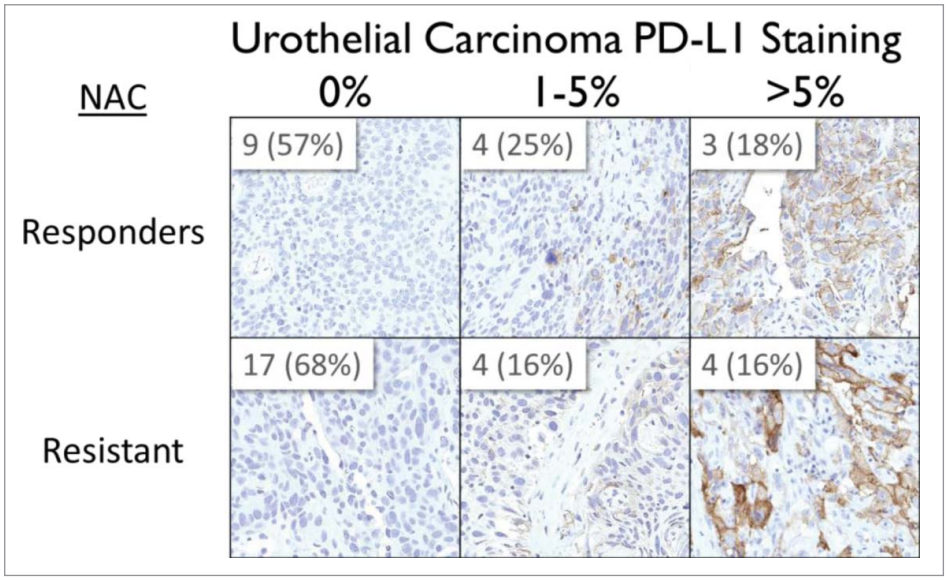

Figure 2. Baseline PD-L1 staining in MIBC for patients treated with platinum-based NAC. Representative images of the spectrum of tumor PD-L1 staining across both cNAC responders and resistant MIBC cases are shown. Included in the top left corner of each image is the total counts and row-wise proportions in the cNAC-treated MIBC cohort. No significant different in baseline tumor PD-L1 staining was observed when comparing cNAC responders and resistant cases (Goodman–Kruskal $p > 0.05$).

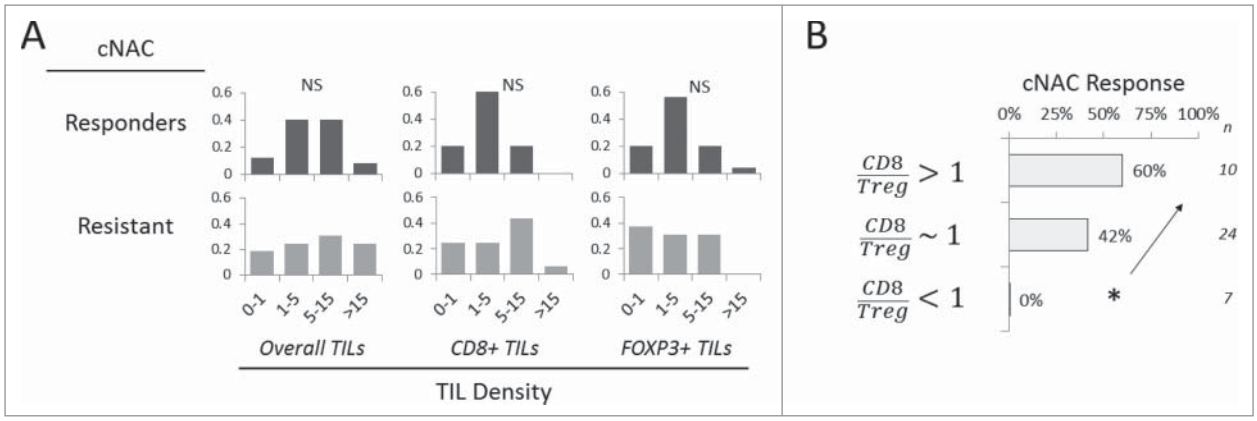

Figure 3. Spectrum of tumor-infiltrating lymphocytes characterized by cNAC response status. (A) Normalized histograms are shown with the TIL densities (TIL counts per 100 tumors cells) binned into the designated intervals on the *x*-axes and the sample proportions shown on the *y*-axes. In each case (overall, CD8, and $FOXP^{3+}$ TILs) no significant (NS, Goodman–Kruskal $p > 0.05$) difference in the TIL density was observed when comparing cNAC responders and resistant cases. (B) The percentage of cases responsive to cNAC is shown on the *x*-axis and the per sample CD8/Treg ratio on the *y*-axes. A striking association is present (*Goodman–Kruskal $p = 0.0003$), in which a CD8 < Treg TIL composition is strongly associated with cNAC resistance and a CD8 > Treg is better associated with cNAC response.

prior to initiation of induction neoadjuvant platinum-based chemotherapy in patients with MIBC. We found that immunohistochemical expression of tumor cell PD-L1 in TUR specimens prior to cNAC was similar (range 16–25%) in those patients whose final pathological stage at the time of cystectomy showed NAC-response (<ypT2) vs. those who were deemed NR (≥ypT2). Although the sample size and specimen sizes (due to TMA format) is limited (n = 41) this cohort represents the only surgical series of which we are aware in which PD-L1 expression could be correlated with pathological outcomes at the time of RC. Given the association between PD-L1 expression and response to PD-1/PD-L1 blocking antibodies in MIBC,[26] these data suggest that PD-L1+ patients could be potential candidates for either anti-PD-1 or PD-L1 antibody therapy in the appropriate clinical context.

Consistent with previous data in MIBC and other tumor types, we observed a strong association between the TIL density and PD-L1 expression, supporting an adaptive immune resistance-mechanism.[15,27] In this MIBC TURBT cohort examined by TMA, less than 10% of cases exhibited significant PD-L1 tumor cell staining in the absence of a significant lymphocytic infiltrate. These findings support the hypothesis that in muscle invasive urothelial carcinoma, PD-L1 expression likely represents "adaptive immune resistance" [10,24,28,29] in PD-L1 expression on tumor cells may be induced by pro-inflammatory cytokines secreted by effector T cells in the tumor.[27,28,30] The 10% of cases in which PD-L1 expression was observed in the absence of a lymphocytic infiltrate may represent "innate immune resistance," in which PD-L1 expression is driven by oncogenic pathways.[28,31] One potential factor confounding these observations could be the influence of prior BCG therapy; a factor which we plan to address more comprehensively in a larger cohort of patients.

The most interesting aspect of our data is the unexpected finding that the CD8 effector to Treg ratio correlates with response to cNAC in MIBC. Patients with a CD8/Treg ratio > 1 had a reasonable response rate, while patients in whom the CD8/Treg ratio was < 1 showed no responses. The biological mechanisms underlying this significant observation are not immediately clear, but our findings are consistent with the notion that the immune system may play an important role in the response to chemotherapy, as has been well-documented in a number of animal models.[32] We examined the CD8 and FOXP3 transcript levels from a recent gene expression profiling dataset of a small cohort of 23 pretreatment MIBC samples[33] subsequently treated with cNAC, analogous to our cohort. In cases where the CD8 transcript out weighted the FOXP3 transcript by a factor of 2 or more, the response rate was 37.5% (3/8) in contrast to the 20% (3/15) for cases in which this did not occur. While this trend is similar to what we observed by IHC in our cohort, the small sample size limits the statistical significance of these findings ($p > 0.05$, Fisher's exact test). Despite the caveats that exist with gene expression profiles of tissue homogenates with varying cellular compositions of tumor, inflammatory, stromal and other cell types, it is encouraging that these mRNA-level data are congruent with our protein-level IHC results. These interesting findings are worthy of further exploration and we plan to examine the relevant additional data as they become available.

The findings must be interpreted with caution, as several caveats exist: First, this was a relatively small patient cohort; results clearly must be verified in larger series. Second, we used FoxP3 staining to quantify Treg, although this is a common practice, we are fully cognizant that FoxP3 may also be expressed on activated $CD4^+$ T cells in humans. Third, we are also aware that we quantified total CD8 T cells, and did not establish effector function by further phenotypic analysis, i.e. by quantifying IFNγ expression using molecular techniques. Finally, these data are only correlative, and it is certainly possible that the increased CD8/Treg ratio may reflect a more genetically unstable tumor with a relatively increased expression of neo-antigens that confer both an "infiltrated" phenotype as well as a greater sensitivity to chemotherapy.

With the completion of the TCGA sequencing efforts in MIBC[34] and a subsequent exome level sequencing in a NAC MIBC cohort,[35] we anticipate that somatic mutations (such as ERCC2, ATM, RB1, FANCC, and others) in urothelial carcinoma will also play a role in identifying patients likely to derive benefit from platinum based NAC in MIBC.[36,37] Furthermore, the relationship of these molecular factors to the current

understanding of variant histologic subtypes[38] along with evolving molecular subtypes of urothelial carcinoma[33,39-43] as it relates to responsiveness to platinum based chemotherapy and the interaction of the host immune remains to be fully characterized.

These intriguing but early results do suggest that the immune system plays a role in the response of MIBC to cNAC. The findings are from a single institution with a relatively smaller sized cohort; certainly, future studies with larger sample sizes and across multiple institutions will be required and are currently being drafted to validate these findings. However, the results in this study would indicate that patients with an unfavorable immune phenotype (i.e., low CD8/Treg ratio) might not be optimal candidates for cNAC, at least with current regimens. Additionally, these findings raise the possibility that immunomodulatory approaches able to tune the host response may be able to better prime patients for response to conventional chemotherapy.

Methods

Clinical cohort

The study was approved by the Johns Hopkins Institutional Review Board. Informed consent was not required because after the data were collected they were analyzed anonymously. We queried the Johns Hopkins Hospital (JHH) Institutional Review Board approved Bladder cancer database to identify patients who received any NAC followed by open RC between 2000 and 2013. Patients with unknown follow-up, cause of death, or documentation of inadequate cNAC dosing were excluded from the cNAC subset described above. The cohort has been previously reported on by our group in two related publications in which further detail on the composition of the cohort can be found.[8,23]

Pathologic assessments

All pathologic evaluations were performed at JHH by an expert GU pathologist. Included patients were required to have histologically confirmed urothelial carcinoma of the bladder diagnosed on pre-operative TURBT biopsy. Patients with variant histologies were included provided the greater than half of the lesion exhibited conventional urothelial morphology. Patients with any small cell histology were excluded. cNAC responders (R) were defined by the absence of residual MIBC (<ypT2) at cystectomy; conversely NR tumors were defined by the presence of residual muscle-invasive (≥ypT2) at cystectomy.

Tissue microarray construction and immunohistochemical assessments

Tissue microarrays were constructed at the Johns Hopkins tissue microarray facility utilizing 1.0 mm cores in triplicate from the same sample, when possible. Antibodies were acquired from commercial sources as implemented as follows. FoxP3 (ebioscience; 14-4777) 1:150 diluted in antibody dilution buffer with an overnight incubation at 4C; 2. CD8 (thermo scientific; RB-9009-P0) 1:800 diluted in antibody dilution buffer with a 45 min incubation at room temp. PD-L1 (Cell Signaling; 13684) 1:100 diluted in antibody dilution buffer with a 45 min incubation at room temp. Detection of immunolabeling was performed using anti-mouse or anti-rabbit HRP-conjugated secondary antibodies and counterstaining was performed with DAB. Antibodies were scored in blinded manner with respect to cNAC response status by a urologic pathologist (ASB). PD-L1 was reported as percent positive in the tumor cells. Similarly, TILs were reported as number of TILs (overall, CD8, or $FOXp3^{+}$) per 100 tumor cells, which we refer to as the TIL density, to best account for variability in tumor cellularity in any given field of view and to be on the same scale as tumor PD-L1 scoring. The aforementioned TIL density (TD) was binned into four intervals: (a). $0 \leq TD < 1$ (b). $1 \leq TD < 5$ (c). $5 \leq TD < 15$ (d). $15 \leq TD < \infty$. These scores were based on a minimum of 100 cells present for evaluation across all available tissue from a given case on the TMAs examined. Additionally, the TIL densities were assigned by comparison to a set of computer generated images modeling tumor and TILs at varying degrees of tumor cellularity and TIL density (Fig. S1).

Statistical analyses

Standard Fisher's exact tests and Goodman–Kruskal tests of association were performed using MATLAB version 8.4 (The MathWorks Inc., Natick, MA, 2000).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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