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Original contribution

Primary adenocarcinoma of the bladder lacks mismatch repair deficiency and demonstrates PD-L1 expression in tumor-infiltrating immune cells, with implications in both diagnosis and therapeutics☆

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Summary Primary adenocarcinoma of the bladder is a rare and highly aggressive disease with no standard therapy. Effectiveness of immune checkpoint blockade in bladder adenocarcinoma is unknown due to lack of clinical trials. Due to the disease rarity, the rate of PD-L1 expression and DNA mismatch repair deficiency is largely unknown. In this study, we examined PD-L1 expression in 56 cases of bladder adenocarcinoma and mismatch repair protein expression in 42 cases of bladder adenocarcinoma using immunohistochemistry. Mismatch repair protein expression was uniformly intact in all cases of bladder adenocarcinoma, in comparison with 19% of advanced colorectal adenocarcinoma being mismatch repair deficient. This finding indicates that mismatch repair proteins may be combined with β-catenin and GATA-3 to create an immunostaining panel which, in addition to clinical studies, can aid in distinguishing bladder adenocarcinoma from secondary involvement by colorectal carcinoma. 4% of cases were found to overexpress PD-L1 in tumor cells while approximately a third of cases were found to display PD-L1 expression in tumor-infiltrating immune cells. These results indicate hypermutators are likely rare in bladder adenocarcinoma, yet 20–30% of the patients may be eligible for immune checkpoint blockade therapy.

1. Introduction

Primary adenocarcinoma of the urinary bladder (BAC) is an aggressive and rare tumor responsible for 0.5–2% of all primary bladder malignancies [1]. Clinical outcome is

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determined by tumor pathological stage, grade, and nodal involvement. Five-year disease-free survival has been estimated to be approximately 50%, which decreases steadily as tumor stage increases [2-4]. Surgical resection remains the standard of therapy for BAC [5-7], and post-operative radiotherapy may have a role in improving disease-free survival, particularly on early stages [4]. However, recurrence is not uncommon, especially in patients with advanced disease. Patients with unresectable or metastatic tumors are often treated by extrapolating data from bladder urothelial carcinoma (BUC) or colorectal adenocarcinoma (CRC). Molecular characterization of BAC by next generation sequencing details tumor heterogeneity and genomic alterations involving the MAP kinase, mTOR, Wnt, and Tp53/Rb1 pathways, which overlap with alterations seen in CRC [8]. The 2019 National Comprehensive Cancer Center Network (NCCN) guidelines for patients with advanced or metastatic BAC recommend chemotherapy including colorectal regimen [9]. However, those chemotherapy regimens have shown poor response rates in BAC [10,11].

Programmed cell death-1 (PD-1) /programmed cell death ligand-1 (PD-L1) blockade immune therapies have successfully attained significant response in cancers from different organ sites [12-14], and in some cases PD-L1 expression in tumor cells has been associated with better response [15]. The effectiveness of checkpoint blockade in BAC is unknown due to lack of clinical trials. In CRC, immunohistochemistry (IHC) for tumor PD-L1 positivity and DNA mismatch repair deficiency (MMRD) was estimated to be 5% and 17%, respectively [16]. Microsatellite instability-high (MSI-H) CRC benefit the most from immune checkpoint blockade therapies, in which PD-L1 overexpression in tumor cells is common [17]. The current 2019 NCCN guidelines for patients with advanced or metastatic CRC recommend nivolumab or pembrolizumab (both anti-PD-1) if tumors are MMRD or MSI-H [18,19]. For BUC, the current 2019 NCCN guidelines recommend atezolizumab (anti-PD-L1) and pembrolizumab (anti-PD1) as first line therapy in patients with locally advanced or metastatic disease for patients whose tumors express PD-L1 or in patients who are not eligible for platinum-containing chemotherapy regardless of PD-L1 expression [9]. Additionally, pembrolizumab is the recommended subsequent therapy (as well as several other PD-1/PD-L1 inhibitors recommended as alternative preferred regimens) in these patients regardless of PD-L1 expression.

The frequency of PD-L1 overexpression and mismatch repair (MMR) status in BAC has been largely unknown. A recent study reported no evidence of MMRD in 15 cases of BAC using IHC [8]. In this study, we aim to evaluate PD-L1 and MMR protein expression in a series of 56 BAC and to provide background data for immune checkpoint blockade treatment in these patients. For pathologists, BAC is often histologically indistinguishable from secondary involvement of bladder by CRC, even with the help of β-catenin and GATA-3 IHC markers [20,21]. As has been reported that 17% of CRC demonstrate MMRD on IHC [16] and 0 of 15 BAC demonstrate MMRD in a recent study [8]; we also aim to explore the usefulness of MMR IHC as a diagnostic tool in distinguishing BAC from secondary involvement by CRC.

2. Materials and methods

2.1. BAC cohort and immunohistochemistry

A total of 56 primary BAC were retrospectively collected from 1987 to 2011 and 3 tissue microarrays (TMA) were constructed with 3–7 cores per tumor as described in previous studies [22,23]. None of the patients were treated with neoadjuvant radiation or chemotherapy. Urothelial carcinoma with glandular differentiation was excluded. 2 TMAs (N = 43) had sufficient tissue and detailed annotations, which included 23 conventional/enteric adenocarcinoma (including 7 mucinous adenocarcinoma), 10 mucinous carcinoma with signet ring cell features, and 10 signet ring cell carcinoma. One case was urachal adenocarcinoma, mucinous type. Of the 43 cases, 22 were transurethral resection or biopsy specimens, and 21 were partial or total cystectomy specimens. Tumors were staged as pT1 (N = 16), pT2 (N = 9), pT3 (N = 10), and pT4 (N = 8), respectively. 6 in 7 cases with known nodal status demonstrated lymph node metastasis. For the third TMA, it included 8 conventional/enteric adenocarcinoma (including 6 mucinous adenocarcinoma) and 5 signet ring cell carcinoma, with other pathologic details not

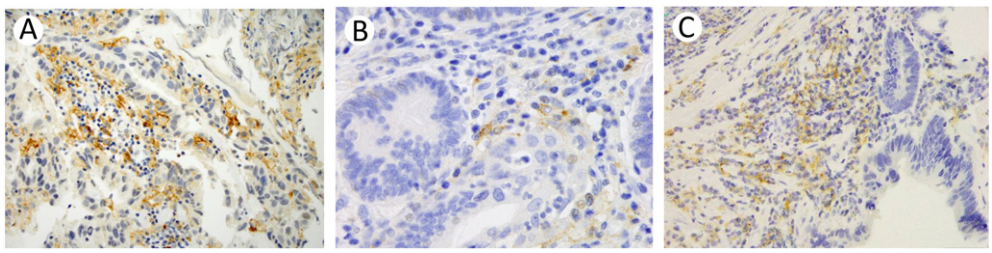

Fig. 1 (A) A representative case of BAC with extensive PD-L1 expression in both tumor cells and tumor-infiltrating immune cells (×400); (**B**) A representative case with focal PD-L1 immunopositivity in tumor-infiltrating immune cells (×400); (**C**) A representative case with extensive PD-L1 immunopositivity in tumor-infiltrating immune cells (×200).

Table 1 PD-L1 expression in BAC.

PD-L1 expression	Tumor cells	Immune cells
0 to< 1%	54/56 (96%)	36/56 (64%)
1 to 4%	0/56 (0%)	4/56 (7%)
5 to 24%	0/56 (0%)	10/56 (18%)
≥25%	2/56 (4%)	6/56 (11%)

Table 2 Binary PD-L1 expression results using criteria for PD-L1 expression in BUC.

PD-L1 expression criteria	Meets	Does not meet
Tumor IC Area (≥5%) [a]	16/56 (29%)	40/56 (71%)
Combined Positive Score (≥10) [b]	11/56 (20%)	45/56 (80%)

[a] Eligibility with treatment with atezolizumab is determined by the tumor-infiltrating immune cells covering ≥5% of tumor area.

[b] Treatment with pembrolizumab is determined by Combined Positive Score of ≥10.

available at the time of manuscript preparation. Tissue from that TMA was only provided for PD-L1 stain.

Five-micron TMA sections were subjected to immunohistochemical analysis. Sections of all 56 cases were stained for anti-PD-L1 (clone E1L3N, rabbit, Cell Signaling; 1:200). Of the two TMAs with sufficient tissue available (N = 43), sections were stained for anti-MSH2 (Biocare Medical, 1:25), anti-MSH6 (BD Biosciences, 1:100), anti-MLH1 (Biocare Medical, 1:20), and anti-PMS2 (BD Biosciences, 1:50). IHC was carried out on the Dako Link 48 autostainer (Agilent), with antibody incubation for 60 minutes, amplification using Envision FLEX rabbit or mouse linkers, and visualization using the Envision Flex High-sensitivity visualization system (Agilent).

PD-L1 positivity was defined by membranous staining. Scoring of PD-L1 expression on tumor cells and tumor-infiltrating immune cells was performed semi-quantitatively as follows: negative (negative or< 1%), minimal (1–4%), focal (5–24%), or extensive (25–100%). Tumor Immune Cell (IC) score was also assigned using a cutoff of ≥5% of the tumor area covered by PD-L1-staining immune cells. Additionally, a Combined Positive Score (CPS) with a cutoff of ≥10 was calculated using manufacturer instructions (100 x [PD-L1 positive tumor cells, lymphocytes, and macrophages] / [total viable tumor cells]). MSH2, MSH6, MLH1, and PMS2 were scored as 0 (no loss) and 1 (complete loss in all tumor cells).

2.2. CRC cohort

Additionally we examined MMR IHC results of a consecutive series of 149 colorectal adenocarcinoma of stage pT3-T4 and/or pM1 at University of California, Los Angeles from 01/01/2016 to 03/15/2019, where an IHC based MMR panel (MSH2, MSH6, MLH1, and PMS2) has been routinely examined and reported in all CRC specimens.

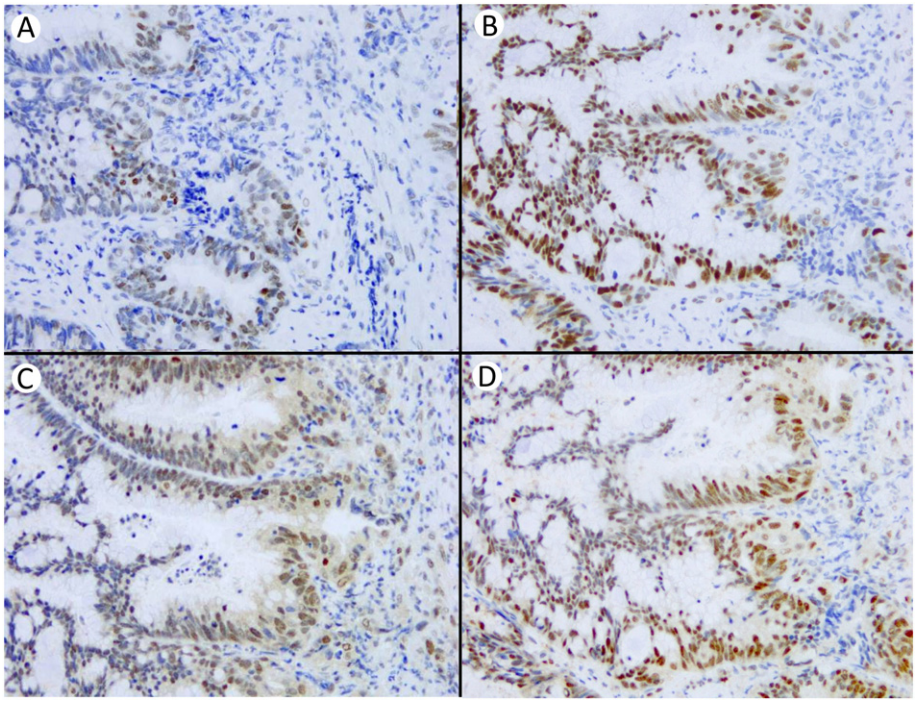

Fig. 2 A representative case of BAC with intact MSH2 (**A**), MSH6 (**B**), MLH1 (**C**), and PMS2 (**D**) expression (×200).

3. Results

3.1. BAC is enriched for PD-L1(+) TILs

Of the total of 56 cases of BAC examined for PD-L1 expression, only 2 cases (4%) displayed increased PD-L1 expression in tumor cells, with both cases showed extensive PD-L1 expression (Fig. 1A). The remaining cases were negative for PD-L1 expression in tumor cells. Examination of tumor-infiltrating immune cells revealed that 4/56 (7%), 10/56 (18%), and 6/56 (11%) cases demonstrated minimal, focal, and extensive PD-L1 expression, respectively (Fig. 1B and C). The PD-L1 expression results are summarized in Table 1. Next, we estimated proportions of BAC patients who may be eligible for immune checkpoint blockade. For CRCs, there is no standardized cutoff to report PD-L1 expression. Some institutions use CPS $\geq$ 1, which is the interpretation criterion for gastric and gastroesophageal junction adenocarcinoma. In contrast, interpretation criteria for PD-L1- positivity has been standardized for BUC: tumor IC score $\geq$ 5% for atezolizumab (Ventana SP142 assay) and CPS $\geq$ 10 for pembrolizumab (22C3 pharmDx) [9]. Using the BUC criteria, we identified that 16/56 (29%) met the cutoff of $\geq$5% tumor area covered by tumor-infiltrating immune cells, and 11/56 (20%) cases met the $\geq$10 cutoff for the CPS (Table 2).

3.2. BAC lacks MMRD

A total of 43 cases of BAC were examined for expression of MMR genes MSH2, MSH6, MLH1, and PMS2. One case was excluded due to suboptimal staining. Of the remaining 42 cases, all had retained MMR gene expression (100%) (Fig. 2).

3.3. 19% of CRC displays MMRD

Review of MMR IHC results of 149 consecutive cases of pT3-T4 and/or metastatic CRC revealed that 28 (19%) of 149 cases were MMR deficient. Compared to 0 of 42 cases of BAC displaying MMRD, CRC has a significantly higher probability of MMRD (P = .002, Chi-square test).

4. Discussion

To our knowledge, this is the first study reporting levels of PD-L1 expression in BAC. While we found rarely BAC overexpressing PD-L1 in tumor cells, approximately a third of cases showed increased expression in tumor-infiltrating immune cells. The lack of MMRD found in 42 cases in this study is consistent with the intact MMR expression in the 15 cases of BAC reported by Roy et al in 2017 [8].

For pathologists, it is often difficult to differentiate primary BAC from secondary involvement of bladder by CRC. The IHC profile of BAC is similar to that of CRC; for example, CK20 and CDX-2 are frequently positive and CK7 is often negative. β-catenin is perhaps the most useful marker so far, as nuclear β-catenin staining is present in approximately 80% of CRC compared to 0–8% cases of BAC [20,24,25]. GATA-3 is generally negative in CRC and expressed in 0–7% of BAC conventional adenocarcinoma subtype [23]. One may use these two markers as a panel, as β-catenin nuclear stain favors CRC and GATA-3 positivity favors BAC (although uncommon). However, frequently pathologists encounter tumors biopsied from bladder that show morphology of enteric type adenocarcinoma are negative for GATA-3 and show no nuclear staining for β-catenin. One still cannot entirely exclude CRC primary, as 20% of CRC can be negative for nuclear β-catenin. We need a discriminatory marker to differentiate the remaining 20% of CRC cases from BAC.

One may compare the genomic profiles of BAC with that of CRC and BUC to look for novel discriminatory IHC markers. In a recent study of targeted next generation sequencing of 15 cases of BAC and 25 cases of CRC, it was found that *TP53* (47%), *PIK3CA* (20%), and *KRAS* (20%) were the most frequently altered genes in BAC, while *CTNNB1* and *APC* gene alterations were restricted to enteric subtype only [8]. BAC shows substantial overlaps in genomic alterations with CRC such as *APC*, *KRAS*, *PIK3CA*, *TP53*, and *CTNNB1*. CRC is divided into microsatellite stable (non-hypermutated) and microsatellite instable (hypermutated) groups [26]. In non-hypermutated CRC, *APC* mutations are significantly more frequently mutated than in hypermutated CRC, indicating MMRD subtype and Wnt pathway activated subtype may be mutually exclusive in CRC [26]. In a later study, CRC was divided into 4 Consensus Molecular Subtypes (CMS) [27]. The CMS2 subtype of CRC (37% of cases) is characterized by Wnt and MYC activation. Most cases of CMS1 subtype (14% of cases) are MMRD (microsatellite instable), while microsatellite instability is seen to a lesser extent in other CMS subtypes [27]. The above genetic classifications are in line with the common immunophenotypes of CRC, of which approximately 80% are positive for nuclear β-catenin, which is a marker of Wnt pathway activation, and approximately 15–20% are MMRD. In this study, 19% of locally advanced or metastatic CRC displayed MMR deficiency on IHC, which is in agreement with the existing data. In summary, the majority of CRCs could be characterized by either nuclear β-catenin expression or MMRD. An immunostaining panel using β-catenin in addition to MMR is likely to detect the majority of CRCs. In contrast to CRC, results from this study (n = 42) and prior work by Roy et al (n = 15) both demonstrated uniformly retained MMR protein expression in BAC. Therefore, β-catenin, GATA-3, and MMR as an immunostaining panel, in addition to clinico-radiological studies, can help in distinguishing BAC from secondary involvement by CRC.

Our cohort only included a limited number of urachal carcinoma, therefore, did not provide direct evidence for the application of this proposed immunostaining panel to

distinguish urachal carcinoma from metastatic CRC. A case series containing 24 urachal carcinoma, including 9 enteric type, found that CK20 and CDX2 were uniformly positive in cases with immunostaining. CK7 had mixed results. β-catenin stain demonstrated cytoplasmic staining in 14 cases and cytoplasmic as well as nuclear staining in only one case [28]. A separate study of 70 cases of urachal carcinoma, including 30 enteric type, demonstrated a universally intact mismatch repair status [29]. These findings suggest an immunohistochemical panel as we propose would also be helpful in differentiating urachal carcinoma with enteric differentiation from CRC.

Therapeutically speaking, our observation that BAC appears to be uniformly intact for the MMR panel indicated that a low probability of BAC would be the MMRD subtype, a subtype most sensitive to checkpoint blockade therapy [30]. MMRD however is only one of several mechanisms of tumor mutagenesis and the overall tumor mutation burden (TMB) must be taken into context when evaluating the potential effectiveness of PD-1/PD-L1 inhibitor therapies. Effectiveness has been associated with a high TMB across a broad range of malignancies and TMB in urothelial carcinoma is generally high, comparable to non-squamous cell carcinoma of the lung [31,32]. The TMB in BAC, which has no data so far, awaits to be investigated. Another predictor for response is overexpression of PD-L1 on tumor cells and/or tumor-infiltrating immune cells. This study indicates that BAC rarely have PD-L1 overexpression in tumor cells, while approximately a third of cases display increased PD-L1 expression in tumor-infiltrating immune cells.

It is yet unknown if PD-L1 expression in BAC correlates with response to immune checkpoint blockade. In studies of patients with metastatic CRC, it was found that PD-L1 expression status (in tumor cells and/ or infiltrating immune cells) was not predictive of therapeutic response to pembrolizumab and nivolumab [33,34]. In BUC, studies regarding the effectiveness of PD-1/PD-L1 inhibitors in urothelial carcinoma based on PD-L1 expression have had mixed results with a general trend towards increased PD-L1 expression in tumor and/or tumor-infiltrating immune cells and a better response to treatment. Rosenburg et al 2016 found that PD-L1 expression in immune cells was associated with a response to atezolizumab [35]. Balar et al 2017 found positive radiological responses to first-line pembrolizumab in a subset patients with cisplatin-ineligible locally advanced or metastatic urothelial carcinoma including patients with absent, low, and high PD-L1 expression in tumor cells and immune cells. It was found that patients with increased PD-L1 expression had a higher frequency of response than those without increased PD-L1 expression [36]. In contrast, in 2017, Balar et al demonstrated a positive responses to atezolizumab as first line treatment for cisplatin ineligible patients with locally advanced or metastatic urothelial carcinoma across all PD-L1 expression subgroups [37]. Additionally Bellmunt et al 2017 found a significant survival advantage in patients treated with second-line pembrolizumab in a study of 542 patients with advanced urothelial carcinoma that was seen regardless of PD-L1 expression status [38]. Similarly, Powles et al 2017 found a positive response to durvalumab (anti-PD-L1) was not related to PD-L1 expression on tumor and/or immune cells [39]. Due to the rarity of BAC, it would be extremely challenging to study the correlations between response and PD-L1 expression in tumor cells and tumor-infiltrating immune cells in this disease. It is reasonable to apply the PD-L1 positivity cut-off criteria in bladder cancer to BAC. Results from this study suggest that 20–29% of patients with late-stage BAC may be eligible for first-line atezolizumab or pembrolizumab and all patients who are ineligible for platinum-containing chemotherapy would be eligible for atezolizumab or pembrolizumab. Consideration of these therapies may be particularly important in BAC since platinum-based chemotherapy regimens have shown very limited responses in BAC and PD-1/PD-L1 inhibitors may offer a comparatively favorable side-effect profile [10,11].

Our study has several limitations. Evaluation of PD-L1 expression using tissue microarray sections may potentially underestimate the level of PD-L1 expression due to tumor heterogeneity. A subset of BAC in this study were of stage pT1, which were suboptimal cases to evaluate PD-L1 expression to guide treatment. We didn't use FDA-approved companion diagnostic kits due to feasibility and lack of evidence from any clinical trials to support the usage of a specific test kit. We chose to use E1L3N clone of anti-PD-L1 to assess PD-L1 positivity, as our prior study demonstrated its equal performance with SP142 and SP263 antibodies [40]. Finally, the TMAs were constructed using tumors from transurethral resection and cystectomy specimens. This cohort may not be the most representative for the target disease population for immunotherapy, which are mostly patients with unresectable or metastatic tumors.

In summary, this study indicates that MMR can be added to β-catenin and GATA-3 to create an immunostaining panel which, combined with clinical studies, can help in distinguishing BAC from secondary involvement by CRC. The presence of MMRD and nuclear β-catenin favors a colorectal primary. 20–29% of BAC meet the criteria for PD-L1 positivity described in the 2019 NCCN guidelines for bladder cancer. PD-1/PD-L1 inhibitors may have a potential role to play in a significant subset of patients with advanced BAC.

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