Immune checkpoints and their inhibitors: Reappraisal of a novel diagnostic and therapeutic dimension in the urologic malignancies

Rohan Sardana
Sourav K. Mishra
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Rohan Sardana a,1, Sourav K. Mishra b,1, Sean R. Williamson c, Abhishek Mohanty d, Sambit K. Mohanty e,*

a Department of Hematopathology, Tata Memorial Hospital, Mumbai, India
b Department of Medical Oncology, Advanced Medical Research Institute, Bhubaneswar, India
c Department of Pathology and Laboratory Medicine, Henry Ford Health System, Detroit, MI, USA
d Principal Research Officer Head of Research, Rajiv Gandhi Cancer Institute and Research Centre, New Delhi, India
e Department of Pathology and Laboratory Medicine, Advanced Medical Research Institute, Bhubaneswar, India

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Advances in molecular immunology have unveiled some of the complexity of the mechanisms regulating cellular immune responses and led to the successful targeting of immune checkpoints in attempts to enhance antitumor T cell responses. Surgery, chemotherapy, and radiation therapy have been the mainstay of treatment in urologic malignancies. Immune checkpoint molecules such as cytotoxic T-lymphocyte associated protein-4, programmed cell death protein-1, and programmed death-ligand 1 have been shown to play central roles in evading cancer immunity. Thus these molecules have been targeted by inhibitors for the management of cancers forming the basis of immunotherapy. Immunotherapy is now among the first line therapeutic options for metastatic renal cell carcinomas. In advanced bladder cancer, immunotherapy is the standard of care in the second line and the first line for cisplatin ineligible patients. There continues to be ongoing research to identify the role if any of immunotherapy in testicular, prostatic, and penile cancers. The ideal biomarker for response to immunotherapy is still elusive. Although programmed death-ligand 1 immunohistochemical testing has been widely used across the globe as a biomarker for immunotherapy, companion diagnostic tests have inherent issues with testing and reporting and cannot have universal applicability. Additional biomarkers including, tumor mutational burden, deficient mismatch repair, high microsatellite instability, and immune gene expression profiling are being evaluated in various clinical trials. This review appraises the data of immunotherapy in the management of urologic malignancies.

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Introduction

Traditionally systemic chemotherapy has been the mainstay of treatment for metastatic malignancies. However, such treatment often has limited efficacy and like most therapies associated toxicities. Although the neoplastic cells are often chemosensitive at the outset with a rapid reduction in the tumor mass, malignant clone(s) may become refractory to the treatment and lead to disease recurrence. Thus, the need to develop and deploy novel therapeutics, and now for nearly two decades there has been increased interest in immunotherapy, specifically checkpoint inhibitors [1].

Urologic malignancies are growths of abnormal cells that form in the organs of the urinary system in both men and women, and in the male genital organs including the testis, prostate, penis, and the glands and ducts associated with these organs. Per GLOBOCAN 2018 data, prostatic malignancies are the third most common cancer worldwide accounting for 1,276,106 cases detected annually with 360,000 deaths. Annually, 550,000 cases of bladder cancer, 400,000 cases of renal cell carcinoma (RCC), 70,000 cases of testicular malignancies, and 35,000 penile cancers are reported; with 200,000 deaths from bladder cancer, 170,000 from RCC 9,000 from testicular malignancies and 15,000 from penile cancers [2].

Until recently, surgery, chemotherapy, hormonal therapy, and radiation therapy were the mainstay of treatment in these malignancies. However, recent advances in genomics, proteomics, and pathway-based analyses have catalyzed and influenced the
landscape of cancer research, fueling the development of novel therapeutics, and deepening our understanding of immunotherapy. As early as the 1990s, Leach et al demonstrated enhancement of antitumor immunity by cytotoxic T-lymphocyte associated protein-4 (CTLA-4) blockade [3]. And in 2003 Phan et al reported on the efficacy of cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma [REF above].

Cancer immunity

The immune system has 2 lines of defense: innate immunity and adaptive immunity. The components of innate immunity include phagocytes (neutrophils, macrophages, dendritic cells, and natural killer [NK] cells), cytokines, and complements. This effectively serves as the first line of defense. T- and B-lymphocytes constitute the principal components of the adaptive or acquired immune system [4,5]. In the context of a malignancy, tumor antigens are presented by the dendritic cells in association with the major histocompatibility complex [MHC] molecules and are recognized by the T-cell receptors (TCR). Thus, dendritic cells act as a bridge between innate and adaptive immunity. The T-cells mount an immune response against cancer in that it is specific (against the tumor antigens), has a memory (accelerated secondary response at recurrence), and adaptive (caters to the tumor heterogeneity) [6,7].

Immune checkpoints

Activation of the immune system is closely regulated to avoid autoimmunity. The activation of T-lymphocytes requires 2 signals: the first signal (signal 1) is received upon presentation by antigen presenting cells [APCs] of the MHC bound processed antigens and polypeptide chain to the TCR [8]. The second signal for activation requires co-stimulation via binding of the molecules B7-1 and B7-2 expressed on APCs to their CD28 and T-cells [9]. This process of T-cell activation and suppression is regulated by various cytokines [10], and by so-called immune checkpoints. Immune checkpoint receptors or molecules are membrane molecules on T-lymphocytes that recognize the ligands on APCs and tumor cells and can play a positive or negative role in the process of the T-lymphocyte activation [11]. Programmed cell death protein (PD-1) and CTLA-4 are the most well studied immune checkpoint molecules expressed on T-lymphocytes [12,13].

CTLA-4-mediated immune checkpoint

CTLA-4 expression and function are intrinsically linked to T-cell activation. Most of the CTLA-4-based T-cell regulation occurs in the peritumoral lymph nodes. It is immediately up-regulated following TCR engagement and competes with the costimulatory molecule CD28 for its ligands, B7-1 (CD80) and B7-2 (CD86) expressed on the APCs [14]. It should be noted that CD80 and CD86 are antigens that are not generally expressed on nonhematological cancer cells but are expressed on APCs, including dendritic cells and monocytes. Since both B7-1 and B7-2 provide positive costimulatory signals through CD28, their binding with CTLA-4 effectively inhibits signal 1 between the TCR and MHC. Thus, it leads to the suppression of T-cell response [14]. Studies by Allison et al showed that administration of monoclonal antibodies against CTLA-4 induced tumor rejection and also led to immunity against secondary exposure to tumor antigens [15]. In 2011, the FDA approved theCTLA-4–blocking antibody, ipilimumab, as the first immune checkpoint inhibitor for the therapy of cancer in metastatic melanoma [16]. As mentioned, the maximum CTLA-4 interaction occurs in the secondary lymphoid organs rather than the T-cell microenvironment, and this has been a major hurdle in finding tumor-specific biomarkers for anti-CTLA-4 response (Fig. 1a).

PD-1/PD-L-mediated immune checkpoint

Another “classical” immune checkpoint molecule, PD-1 interacts with its ligands PD-L1 and PD-L2. These are constitutively expressed at moderate levels in several nonlymphoid tissues, such as heart, lungs, and placenta (responsible for playing a critical role in fetal-maternal tolerance) and are also induced by inflammatory signals [17]. In the setting of immune activation and inflammation, PD-1 expression is induced on the CD4+ and CD8+ T-cells, B-cells, NK cells, NKT cells, macrophages, and some dendritic cells. The interaction of PD-L1 with PD-1 triggers PD-1 phosphorylation and recruitment of the SHP2 phosphatase. PD-1-associated SHP2 dephosphorylates CD28 and TCR signaling to inhibit the T-cell response restricting over reactive T-cell and hence autoimmune [18]. Binding of PD-L1 expressed on the tumor cells to PD-1 leads to inhibition of the signal 1 between the TCR and MHC. It thus inhibits T-cell activation. Unlike, CTLA-4, the interaction between PD-1 and PD-L1 occurs on the tumor cells itself (Fig. 1b) [17,18].

Other immune checkpoint molecules

Further studies into the T-cell costimulatory molecules have revealed several proteins belonging to multiple structurally defined superfamilies. Among these molecules are LAG3, TIM3, TIGIT, VISTA, and ICOS from the immunoglobulin superfamily and OX40, GITR, 4-1BB, CD40, and CD27 from the tumor necrosis factor receptor superfamily etc. However, we lack the fundamental knowledge regarding the biological roles of these molecules. There are many additional costimulatory molecules of uncertain therapeutic value, including newly identified B7 ligand family members [19–24]. The various immune checkpoint molecules, their ligands, expression and biological functions are described (Table 1).

PD-L1 assay by IHC

Antibody clones

Different PD-L1/PD-1 antibodies have been approved for use as biomarkers for immune checkpoint blockade in various malignancies, including the genitourinary malignancies as illustrated in Table 2. The clones approved and tested commonly include 22C3, 28-8, SP142, SP263, and E1L3N [25–28]. These clones have been validated and compared in numerous studies, where Fleiss kappa and intraclass correlation coefficient analyses showed excellent agreement and reliability among all antibodies [29–31]. The main difference between these antibodies lies primarily in their different scoring algorithms.

Positive PD-L1 staining expresion is defined as complete and/or partial, circumferential or linear plasma membrane staining at any intensity that can be differentiated from the background and diffuse cytoplasmic staining. Certain specific terminologies are universally used during the evaluation and reporting of PD-L1, which are as follows: TC: entire tumor cell; IC: entire immune cell; PTC: PD-L1-positive tumor cell; PIC: PD-L1-positive immune cell (Figs. 2 and 3). The scoring for various clones in urologic malignancies is described below.

SP142: PIC/(TC+PTC) 22C3: Combined Positive Score (CPS) = ((PTC+PIC)/(TC+PTC)) x100 SP263: The tumor cell score (TC-Score) = PIC/(IC+PIC) or PTC/(PTC+TC) 28-8: Tumor Proportion Score (TPS) = Percentage of viable tumor cells showing partial or complete membrane staining.

Points to be considered while evaluating biomarkers of immune checkpoint blockade [32]
1. It is advised to use freshly cut tissue samples, as stored slides may yield improper results. Sample fixation in 10% neutral buffered formalin is preferred. There appears to be no effect of delayed fixation on PD-L1 immunohistochemistry (IHC) [33]. However, these findings need to be further substantiated. Decalcification performed with Ethylene diamine tetra acetic acid (EDTA) or acid decalifier lowers the proportion and intensity of the stained cells with PD-L1 using the 22C3 clone, but not in the E1L3N clone [33].

2. Most of the immune checkpoint inhibitors are approved for use in invasive tumors, thus it is mandatory to have part of the invasive component of the tumor in the section on which biomarkers are being evaluated. If initially only a superficial biopsy was provided, it is advised to request a fresh biopsy with adequate invasive component for evaluation.

3. At least 100 viable tumor cells are required for evaluation. Avoid areas with extensive necrosis and hemorrhage.

4. Tonsil is used as a positive control as the crypt epithelium shows strong PD-L1 staining. Additionally, the immune cells including macrophages, lymphocytes, and dendritic cells within the tonsil exhibit an intermediate to strong cytoplasmic or membranous staining.

**Role of immune checkpoint inhibitors in urothelial carcinoma of the bladder**

Bladder cancer is the 11th most common cancer worldwide accounting for 549,000 new cases annually. There is a male predilection and it is the 14th leading cause of cancer mortality per GLOBOCAN 2018 data [2]. Urothelial carcinoma of the bladder ac-
Table 1

<table>
<thead>
<tr>
<th>Checkpoint molecules</th>
<th>Ligand(s)</th>
<th>Expression pattern</th>
<th>Cellular mechanism</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA4 [15]</td>
<td></td>
<td>T-cells particularly T-regulatory cells</td>
<td>Competitive inhibition of CD28 co-stimulation</td>
<td>Inhibition of T-cell</td>
</tr>
<tr>
<td>PD-1 [18]</td>
<td>PD-L1, PD-L2</td>
<td>T-cells, Natural killer cells, B-cells, Macrophages, Subsets of dendritic cells</td>
<td>Attenuate proximal T-cell receptor signaling</td>
<td>Inhibition of T-cell</td>
</tr>
<tr>
<td>LAG3 [22]</td>
<td>MHC II, LSECtin</td>
<td>T-cells, Natural killer cells, T-regulatory cells</td>
<td>Mechanism unknown</td>
<td>Negative regulator of T-cell expansion</td>
</tr>
<tr>
<td>TIM3 [23]</td>
<td>Galactin-9, HMGBl, CEACAM-1</td>
<td>Th1 CD4, T-cell, T-regulatory cells, Dendritic cells, Natural killer cells, Monocytes</td>
<td>Negative regulation of proximal T-cell receptor components</td>
<td>Negative regulation of type 1 immunity</td>
</tr>
</tbody>
</table>

CEACAM-1 = carcinoembryonic antigen-related cell adhesion molecules-1; CTLA-4 = cytotoxic T lymphocytes Antigen-4; HMGBl = high mobility group protein 1; LAG3 = lymphocyte-activation gene 3; LSECtin = liver and lymph node sinusoidal endothelial cell C-type lectin; MHC = major histocompatibility complex; PD-1 = programmed death receptor 1; PD-L1 = programmed death receptor ligand 1; PVR = poliovirus receptor; PVRL2 = poliovirus receptor-related 2; Th = T-helper cell; Tc = type 1 CD8+ T-cells; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIM3 = T-cell immunoglobulin mucin-3.

Table 2

<table>
<thead>
<tr>
<th>Antibody clone</th>
<th>Staining platform</th>
<th>Staining characteristics</th>
<th>Drug for which the clone is approved</th>
<th>Positivity criteria</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>22C3 [25]</td>
<td>DAKO</td>
<td>Homogenous membranous tumor cell staining, Mostly weak staining intensity</td>
<td>Pembrolizumab [anti-PD-1]</td>
<td>CPS &gt; 10</td>
<td>CPS includes immune cells and tumor cells</td>
</tr>
<tr>
<td>28-8 [26]</td>
<td>DAKO</td>
<td>Homogenous membranous tumor cell staining, Strong staining intensity</td>
<td>Nivolumab [anti-PD-1]</td>
<td>TPS &gt; 1%</td>
<td>Plasma cells and neutrophils need to be excluded from scoring</td>
</tr>
<tr>
<td>SP142 [27]</td>
<td>Ventana</td>
<td>Dot like staining pattern, Low tumor cell staining</td>
<td>Atezolizumab [anti-PD-L-1]</td>
<td>≤ 5% tumor cells are positive</td>
<td>All immune cells (including granulocytes) apart from the plasma cells are included in the scoring</td>
</tr>
<tr>
<td>SP263 [28]</td>
<td>Ventana</td>
<td>Homogenous cytoplasmic and membranous tumor cell staining, Mostly strong staining intensity</td>
<td>Durvalumab [anti-PD-L-1]</td>
<td>≥ 25% tumor immune cell area and/or 25% of tumor cells</td>
<td>Immune cell positivity is scored according to the area occupied by all immune cells.</td>
</tr>
<tr>
<td>E1L3N [29]</td>
<td>Cell Signaling Technologies</td>
<td>No data sheet available</td>
<td></td>
<td>Used as ≥ 25% in studies</td>
<td></td>
</tr>
</tbody>
</table>

CPS = combined positive score; TPS = tumor proportion score.

counts for ≈95% of bladder tumors with ≈70% of patients presenting with nonmuscle disease [34]. These patients are treated with localized therapies including transurethral resection of the bladder tumor and adjuvant intravesical agents like Bacillus Calmette–Guérin or intravesical chemotherapy. For patients with recurrent Bacillus Calmette-Guérin-unresponsive high-risk carcinoma in situ with or without papillary tumors who are either unwilling/ineligible for cystectomy, pembrolizumab may be a treatment option based on high rates of complete (38.8%) and durable responses (median 14 months) [35,36]. The mainstay of treatment in muscle invasive bladder cancer (MIBC) is radical cystectomy. Based on preference, patients may be treated with neoadjuvant or adjuvant cisplatin-based chemotherapy. Neoadjuvant cisplatin-based chemotherapy regimens are associated with pathological complete responses (pCR) of ≈30% and attainment of pCR is associated with improved survival [37–39]. The role of immunotherapy in MIBC in
the neoadjuvant or adjuvant setting is an area of intense research [40]. The PURE-01 study included 114 patients with a new diagnosis of MIBC and any histology, including 19 patients with predominant variant histology (VH) defined as involving >50% of the tumor specimens, who were treated with 3 cycles of neoadjuvant pembrolizumab prior to radical cystectomy. The primary endpoint was pathological complete response (pT0) in the intent to treat population. The pT0 rate in the ITT population was 37% with responses seen even in the VH. Six of the 7 patients with predominant VH presented with squamous cell carcinoma and 6 of the 7 had downstaging to pT1 with one pT0, 2 of 3 lymphoepitheliomalic variants had a pT0 response. None of the remaining 9 predominant VHs had a response. TMB and CPS were predictors of pT0 irrespective of histology. Pembrolizumab was associated with few immune-related adverse events and did not delay planned surgery, nor increase postsurgical complications [41,42].

In the phase II ABACUS study including 95 patients with MIBC, 2 cycles of neoadjuvant atezolizumab led to pathological complete response in 31% of patients. Baseline biomarkers showed pre-existing activated T-cell expression correlated with outcome whereas unlike findings in the metastatic setting, TMB was not predictive of outcome [43–44]. Studies have shown the feasibility of combining gemcitabine and cisplatin chemotherapy with pembrolizumab or nivolumab in the neoadjuvant setting with pCR rates of 44% and 49%, respectively [45–46].

Cisplatin-based chemotherapy is the treatment of choice in patients with metastatic urothelial carcinoma of the bladder. Overall response rates (ORRs) range from 60% to 70%, overall survival (OS) from 14 to 15 months, and 5-year survival from 13% to 15% [47]. In patients who relapse after platinum-based chemotherapy, the ORR ranges from 5% to 29% with a median OS of 6.9 months based on clinical trials of second-line chemotherapy with paclitaxel and vinflunine [48]. The clinical trials involving immune checkpoint inhibitors in the management of urothelial carcinoma of the bladder are summarized in Table 3 [49–57].

For practical purposes, combination cisplatin-based chemotherapy remains the standard of care in the treatment of metastatic urothelial carcinoma. This includes patients with urothelial carcinoma of the bladder, ureter, renal pelvis, and urethra. In patients eligible for cisplatin, atezolizumab with or without chemotherapy was studied in the IMVigor 130 trial in frontline metastatic urothelial carcinoma. Although there was a statistically significant improvement in the progression-free survival (PFS) in the experimental arm, the OS data are still immature [49]. Based on these results, at the present time combination immunotherapy plus chemotherapy should not be considered as the standard of care in frontline metastatic urothelial carcinoma. Cisplatin ineligibility

Fig. 2. Diagrammatic representations of certain specific terminologies used during the evaluation and reporting of PD-L1 (TC: entire tumor cell; IC: entire immune cell; PTC: PD-L1-positive tumor cell; PIC: PD-L1-positive immune cell).

Fig. 3. (A) Metastatic clear cell renal cell carcinoma (hematoxylin and eosin, original magnification x200). (B) Strong and linear incomplete to circumferential PD-L1 staining in 70% of the metastatic clear cell renal cell carcinoma cells (SP-263 PD-L1 immunohistochemistry, original magnification x400). (C) Metastatic urothelial carcinoma (hematoxylin and eosin, original magnification x200). (D) Strong and circumferential PD-L1 staining in 90% of the metastatic urothelial carcinoma cells (SP-263 PD-L1 immunohistochemistry, original magnification x400).
Table 3  
Clinical trials involving the immune checkpoint inhibitors in urothelial carcinomas.

<table>
<thead>
<tr>
<th>ICI</th>
<th>Study</th>
<th>Phase of study</th>
<th>Reference</th>
<th>Design Eligibility Criteria</th>
<th>Companion Diagnostic Kit</th>
<th>Study arm</th>
<th>Outcomes</th>
<th>Toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pembrolizumab</td>
<td>KEYNOTE 052</td>
<td>Phase 2</td>
<td>O' Donnell et al [38]</td>
<td>First-line UCa; Ineligible for platinum-based chemotherapy</td>
<td>CPS assessed by IHC 22C3 pharmDX assay [DAKO]</td>
<td>Single arm: Pembrolizumab 200 mg q3 weeks for 2 years</td>
<td>ORR 29%; CPS ≥10; 47%; CPS &lt;10: 20%; 30% had CPS ≥10</td>
<td>Grade ≥3 AEs, 21%</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Keynote 045</td>
<td>Phase 3</td>
<td>Bellmunt et al [39]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>CPS assessed by IHC 22C3 pharmDX assay [DAKO]</td>
<td>Pembrolizumab 200 mg q 3 weeks X 24 months vs Investigator's choice paclitaxel, docetaxel, or vinflunine</td>
<td>OS: 10.1 vs 7.2 months (HR: 0.79 95% CI 0.57–0.85 P = 0.0003)</td>
<td>Grade 3 AE, 17 vs 50%</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Keynote 045</td>
<td>Phase 3</td>
<td>Bal et al [40]</td>
<td>First-line UCa; Ineligible for platinum-based chemotherapy</td>
<td>IC assessed by SP142 assay [Ventana]</td>
<td>Atezolizumab 1,200 mg q3 weeks until progression</td>
<td>ORR 23%</td>
<td>Responses irrespective of IC</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Keynote 130</td>
<td>Phase 3</td>
<td>Grande et al [41]</td>
<td>First-line advanced/metastatic UCa</td>
<td>IC as assessed by SP142 assay [Ventana]</td>
<td>Atezolizumab1200 mg q3 weeks vs Investigator's choice of paclitaxel, docetaxel, or vinflunine</td>
<td>ORR: Arm A 8.2 months</td>
<td>Responses irrespective of IC</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Keynote 130</td>
<td>Phase 3</td>
<td>Powles et al [42]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>IC as assessed by SP142 assay [Ventana]</td>
<td>Atezolizumab1200 mg q3 weeks vs Investigator's choice of paclitaxel, docetaxel, or vinflunine until progression</td>
<td>PFS: - Arm A 11.1 vs 10.6 months; NS</td>
<td>Rx withdrawal due to AE: - Arm A 34%</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>CheckMate 275</td>
<td>Phase 2</td>
<td>Sharma et al [43]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>Tumor cell staining ≥1% or ≥5% assessed by IHC 28-8 pharmDX assay [DAKO]</td>
<td>Nivolumab 3 mg/kg q2 weeks until disease progression</td>
<td>ORR 16.6%</td>
<td>No difference in ORR between PD-L1 IHC ≥5% or &lt;5%</td>
</tr>
<tr>
<td>Nivolumab+</td>
<td>Ipi-012</td>
<td>Phase 2</td>
<td>Sharma et al [44]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>Tumor cell staining ≥1% assessed by IHC 28-8 pharmDX assay [DAKO]</td>
<td>Nivolumab 3 mg/kg q2 weeks until disease progression</td>
<td>ORR: Nivolumab+IPI 25.6%</td>
<td>Discontinuation rate due to AE, 7% vs 18%</td>
</tr>
<tr>
<td>Avelumab</td>
<td>Javelin solid tumor</td>
<td>Phase 1</td>
<td>Patel et al [45]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>Tumor cell staining ≥5% assessed by IHC73-10 pharmDX assay [DAKO]</td>
<td>Avelumab 10 mg/kg q2 weeks until progression</td>
<td>ORR: Avelumab 17%; PD-L1+ 24%; PD-L1− 14%</td>
<td>Discontinuation rate due to AE, 7% vs 18%</td>
</tr>
<tr>
<td>Durvalumab</td>
<td>Study 1108</td>
<td>Phase 1/2</td>
<td>Powles et al [46]</td>
<td>UCa that recurred or progressed after platinum-based chemotherapy</td>
<td>SP263 assay [Ventana]</td>
<td>Durvalumab 10 mg/kg q2 weeks until progression</td>
<td>ORR: Durvalumab 18%; PD-L1 high 28%; PD-L1 low 5%</td>
<td>Grade 3 AEs, 7%</td>
</tr>
</tbody>
</table>

AE = adverse events; CI = confidence interval; CPS = combined proportion score defined as number of PD-L1 staining cells (tumor cells, immune cells) divided by the total number of viable tumor cells, multiplied by 100; DOR = duration of response; HR = hazard ratio; IC = immune cells (PD-L1 expression on tumor-infiltrating immune cells); ICI (PD-L1 expression on ≥1% of IC); ICI (PD-L1 expression on ≥1% and <5% of IC), or ICI2/3 (PD-L1 expression on ≥5% of IC); ICI = immune checkpoint inhibitor; IHC = immunohistochemistry; OR = overall response rate; OS = overall survival; PD-L1, programmed death ligand 1; PD-L1− (negative), ≥5% tumor cells with any intensity of PD-L1 expression in plasma membrane; PD-L1+ (positive), ≥5% tumor cells with any intensity of PD-L1 expression in plasma membrane; PD-L1 “high,” ≥25% of either TCs or immune cells staining for PD-L1; PD-L1 “low or negative,” <25% of both TCs and immune cells staining for PD-L1; Platinum-ineligible (ECOG PS 2, CrCl ≥30 to <60 ml/min, grade ≥2 neuropathy/hearing loss, NYHA Class III heart failure)/UCa = urothelial carcinoma.

Treatment Regimens: NIVO 3 = nivolumab 3 mg/kg monotherapy every 2 weeks; NIVO+IPI = nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 weeks for four doses followed by nivolumab monotherapy 3 mg/kg every 2 weeks; NIVO+IPI3 = nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every 3 weeks for four doses followed by nivolumab monotherapy 3 mg/kg every 2 weeks.
is defined as an Eastern Cooperative Oncology Group performance status greater than 2; a creatinine clearance less than 60 mL/min; neuropathy/hearing loss grade 2 or higher; and New York Heart Association heart failure grade 3 or higher [50,51]. Patients ineligible for cisplatin can be considered for frontline immunotherapy. Pembrolizumab (Keynote 052) and atezolizumab (IMvigor 210) have shown ORRs of 23%–29% and a manageable toxicity profile in such patients. They have been approved by the FDA for this indication based on superior overall response rates in comparison to historical ORRs of 10% [50,51]. In the second line, although the FDA has approved all 5 immune checkpoint inhibitors, the data is strongest for pembrolizumab (Keynote 045), which showed a statistically significant improvement in survival compared to standard chemotherapy [52]. Atezolizumab did not show any survival benefit although it was well tolerated and the duration of response was longer [53]. In an analysis of 120 patients with metastatic urothelial carcinoma who received single-agent PD-1/PD-L1 inhibition, 28 of whom had mixed histology, pure versus mixed urothelial histology was not predictive of a differential response (hazard ratio [HR] 1.52, 95% confidence interval [CI] 0.59–3.98, P = 0.39) suggesting that immunotherapy can be used in metastatic urothelial carcinoma with pure or mixed histology [58].

**Role of immune checkpoint inhibitors in RCC**

Renal tumors account for about 403,000 new cases each year and are the 15th most common cancers in the world [2]. Clear cell carcinoma is the most common pathological subtype and accounts for 70% of all RCCs. Nonclear cell carcinomas include mainly papillary and chromophobe RCCs. Approximately 2–3% of all RCCs are hereditary. Several tumor suppressor genes involved in the development of RCC including **PBRM1, BAP1** (BRCA1-associated protein), and **SETD2**, are located in the short arm of chromosome 3 where the VHL gene is located [59]. Studies have shown RCC to be an immunogenic tumor [60]; however, tumor induced immunosuppression is frequent in these patients. This is mediated by multiple mechanisms among which regulatory T lymphocytes and myeloid-derived suppressor cells have been most widely studied. CD4+CD25+Foxp3+regulatory T-cells are responsible for maintaining immune homeostasis. A subpopulation of these cells is expanded in the blood and tumor of patients with RCC. Their expansion correlates with a poor prognosis [61]. These cells cause immunosuppression by producing the IL-10 and transforming growth factor (TGF-β) cytokines which inhibit dendritic cells, ultimately leading to suppression of effector T-lymphocytes [62]. Studies in patients with RCC have also shown that CD4+T-cells tend to express a naïve/resting phenotype [63]. Based on the above knowledge, trials were initially designed to combat the suppression imposed by regulatory T-cell. PD-L1 expression is associated with a poor prognosis in RCC presumably because of its immunosuppressive function. Interleukin-2 immunotherapy has been shown to induce long lasting complete remissions, albeit in only 5%–7% of patients with advanced RCC [64]. It has been postulated that anti PD-1 therapy will restore antitumor immunity and improve survival and this encouraged research into immunotherapy targeting the PD-1–PD-L1 pathway to reinvigorate the tumor specific T-cell mediated immunity in RCC. One of the first trials was a phase II study of single agent CTLA-4 inhibitor, ipilimumab [65]. Based on results from the Checkmate 025 trial which evaluated nivolumab in patients with advanced RCC refractory to inhibitors of vascular endothelial growth factor (VEGF) pathway, nivolumab became the first immunotherapy to be approved for this indication [66]. Given that both the VEGF and PD-L1 pathways are important in the pathogenesis of RCC, it followed that concurrent inhibition of VEGF signaling might enhance the efficacy of immunotherapy in the front-line treatment of patients with metastatic RCC. Results from studies combining this approach support this hypothesis and these are summarized in Table 4 [66,68–71]. With a plethora of new drugs approved in the front line, there is no clear indication as to which regimen to choose. Based on the available data, the National Comprehensive Cancer Network (NCCN) kidney cancer panel has listed nivolumab+ipilimumab (CheckMate 214) and axitinib+pembrolizumab (Keynote 426) as category 1 treatment options for first-line treatment of intermediate- and poor-risk metastatic RCC. Axitinib+avelumab (JAVELIN Renal 101) is also included but not as a preferred option. For the favorable risk subset, the NCCN panel recommends axitinib+pembrolizumab (Keynote 426) as one of the preferred options along with single agent anti-VEGF tyrosine kinase inhibitors (TKIs). Nivolumab+ipilimumab (CheckMate 214) and axitinib+avelumab (JAVELIN Renal 101) are other therapeutic options. For patients with progression on first-line TKIs, nivolumab (CheckMate 025) is the category 1 recommendation. Based on data in frontline, nivolumab +ipilimumab may also be considered as a preferred option although there are no data for this combination in second line. Axitinib+pembrolizumab and axitinib+avelumab are other options [67]. The recommendations made above are based on clinical trials in tumors with clear cell histology. Most studies have excluded patients with nonclear cell RCC. The NCCN panel recommends anti-VEGF TKIs as the preferred option in this subset of patients [67]; Of special mention, the combination of bevacizumab+atezolizumab (IM motion 151) showed a PFS benefit in the front line treatment of metastatic clear cell and sarcomatoid RCC and can be considered in these subsets of patients [71].

**Role of immune checkpoint inhibitors in prostate cancer**

Traditionally, androgen deprivation therapy (ADT) is the cornerstone of management of metastatic prostate cancer (PC). It has been observed that ADT sensitizes the neoplastic cells to the patient’s cell mediated immunity. Thus, combination of ADT with immunotherapy is being explored in many clinical trials [72]. To date, immune checkpoint inhibitors have demonstrated only very marginal benefit in PC, particularly in metastatic castrate resistant. This is possibly due to dysfunctional cell-mediated immunity (defective functioning of circulating T-cells and NK cells) and an immunosuppressive tumor microenvironment (TME) with a preponderance of immunosuppressive regulatory T-cells and myeloid derived suppressor cells in the TME [73,74]. A few studies have indicated that the presence of increased tumor infiltrating (TILs) CD8+T-cells is associated with a poor prognosis [75–77] and some have suggested that TILs in PC may be dysfunctional or may have undergone anergy, exhaustion, or senescence, leading to suppressed T-cell functioning even after the administration of immune checkpoint inhibitors [26,78]. In 2010, the FDA approved the first dendritic cell-based vaccine (sipuleucel-T) as the only immunotherapeutic option for asymptomatic mCRPC. There are 50 ongoing clinical trials involving PD-1/PD-L1 inhibitors and 19 trials with CTLA-4 inhibitors, either as single agents or as part of combination therapy in patients with metastatic prostate cancer [72]. In one that administered a combination a PC vaccine, GVAX, and ipilimumab to men with mCRPC, Prostate specific antigen (PSA) responses were seen in a quarter of the patients [79]. A combination of chemotherapy with immunotherapy has also been explored, the concept being immunologic cell death during chemotherapy releases neoantigens and damage associated molecular patterns potentiating local immune responses [80]. However, 2 phase 3 trials of the anti CTLA-4 antibody ipilimumab failed to show an OS benefit in patients with mCRPC whose tumors were either chemotherapy-naive or had progressed on chemotherapy.
with only a slight improvement in PFS and a modest biochemical response, neither significant [81,82]. A combination of ipilimumab and nivolumab reported responses in a quarter of patients with asymptomatic mCRPC whose tumors had progressed after second-generation hormonal therapy and were chemotherapy-naïve. In patients whose tumors had been exposed to axaban-based chemotherapy, the ORR was 10% [83]. In a phase 1 trial in patients with mCRPC, the ORR with pembrolizumab was 17% and 34% of patients had stable disease [84]. Two retrospective analyses have demonstrated benefit from ICIs in PC harboring a germline or somatic mismatch repair deficiency (dMMR) or microsatellite instability high (MSI-H) genetic signature. In one study conducted at the Memorial Sloan Kettering Cancer Center, dMMR/high microsatellite instability (MSI-H) was detected in 3.1% of metastatic CRPC. Eleven patients were treated with anti-PD-1/PD-L1 therapy, and 6 (54.5%) of these patients had greater than 50% reduction in PSA levels with 4 additionally having radiographic responses. Five (45.5%) patients overall were continuing to respond at 89 weeks [85]. In a smaller study, PC with dMMR was demonstrated to be particularly sensitive to both hormonal therapy (PFS 67 months to initial ADT) and aneclastic responses to pembrolizumab with a PFS of 9 months and PSA responses in 2 out of 4 patients [86]. Finally, pembrolizumab has demonstrated a durable response in a treatment refractory PC with high mutational burden due to DNA polymerase epsilon (POLE) mutation, even though the patient’s tumor was shown to be microsatellite stable. This provides some
support for the concept for POLE mutations as a biomarker for response to immunotherapy, although the magnitude of the benefit and the frequency with which it might be observed has not been defined and is not expected to be high [87]. To summarize, the benefit of immune checkpoint inhibitors in metastatic PC has been very minimal unlike that observed in urothelial carcinoma and RCC. This is an area of active and ongoing research.

Role of immune checkpoint inhibitors in testicular tumors

The testes are immunologically privileged sites. Germ cell tumors (GCT) account for majority of testicular tumors and are subdivided into seminoma and nonseminomatous subtypes. The primary therapeutic modality is orchectomy followed by platinum-based chemotherapy. Ciera et al have confirmed a lack of PD-1 expression in testicular GCT, along with higher levels of PD-L1 on the surface of neoplastic cells, compared to the adjacent uninvolved testis [88]. Chovanec et al have demonstrated that patients whose GCTs have higher levels of PD-L1 expression on the TILs have better PFS and OS [89]. In another study, seminomas had low expression of PD-L1 on tumor cells, with higher expression on TILs, whereas teratomas had high expression of PD-L1 on tumor cells, but low expression on TILs. Among the GCTs, seminomas have the highest frequency of PD-L1 positive TILs (95.9% of cases), followed by embryonal carcinomas (91.0%), yolk sac tumors (60%), choriocarcinomas (54.5%), and teratomas (35.7%) [90]. One of the other factors that modify the PD-L1 expression is the wingless-related integration site signaling pathway, which results in a low number of TILs and reduces the effectiveness of immune checkpoint inhibitors. The wingless-related integration site signaling pathway is commonly activated in nonseminomatous GCTs compared to seminomas [91–93]. Immune checkpoint inhibitors have rarely been used in platinum refractory GCTs. Tumor regression was reported in one case of metastatic embryonal carcinoma treated with nivolumab [94]. Zschabitz et al published their experience on 7 platinum-refractory nonseminomatous germ cell tumors that were treated with nivolumab or pembrolizumab. In 3 of the 7 patients, the tumors displayed some amount of tumor regression. However, there was no association between the PD-L1 staining and the response to therapy [95]. A phase 2 clinical trial in relapsed GCTs reported a clinical response in 2 of 12 patient treated with pembrolizumab [96].

Role of immune checkpoint inhibitors in penile carcinoma

Penile squamous cell carcinomas are rare malignancies and the prognosis with metastatic disease is dismal. Cisplatin- and paclitaxel-based chemotherapy is the mainstay of treatment in advanced penile squamous cell carcinomas. Newer therapeutic modalities are an unmet need in this neoplasm. None of the immune checkpoint inhibitors are currently approved in the treatment of advanced penile squamous cell carcinomas. A few studies have shown high expression of PD-L1 on tumor cells and a correlation of this expression with nodal metastases and an overall poor survival. In a retrospective analysis using an indigenously developed anti PD-L1 antibody (Clone 5H1), Udager et al have demonstrated PD-L1 positivity (defined as membranous staining in ≥5% of tumor cells) in 62% of the primary tumors [97]. PD-L1 expression was associated with higher regional nodal involvement and reduced cancer specific survival. Interestingly there was no significant expression of PD-L1 on immune cells in the tumor microenvironment. In another study using the rabbit monoclonal anti-PD-L1 antibody, cell signaling, E1L3N, 40% of penile squamous cell carcinomas expressed PD-L1 defined by the presence of any membranous positivity on tumor or immune cells in one or more representative spots. As described in the previous study, PD-L1 expression correlated with higher tumor stage and nodal involvement [98]. Per both the studies, immune checkpoint inhibitors should be explored as a possible treatment option for penile squamous cell carcinomas. Pembrolizumab is approved across many solid malignancies with MSI-H. However, there is a paucity of data on PD-L1 in the penile squamous cell carcinomas. By using tumor mutational burden as a surrogate marker for MSI (MSI-H tumors have high mutational load), 6% of penile squamous cell carcinomas were shown to harbor a mutational burden of 20/megabase or greater. In contrast to other neoplasms where high mutational load is a marker of MSI-H status, in penile squamous cell carcinomas this finding probably results from POLE mutations rather than mismatch repair deficiency [99,100]. Although POLE and DNA polymerase delta 1 (POLD) mutations have been shown to be predictive of response to ICIs across varied cancer types including bladder and prostatic cancers independent of MSI status, it remains to be proven whether the same can be extrapolated to penile squamous cell carcinomas [101].

Biomarkers to assess the efficacy of immune checkpoint inhibitors

**PD-L1 expression**

PD-L1 is expressed in 20–30% cases of metastatic urothelial carcinoma [102]. In bladder cancer it is both a prognostic (increased expression correlates with advanced stage and worse outcomes) and predictive marker for response to anti-PD-1 and -PD-L1 therapy [103]. The benefits from the immune checkpoint inhibitors occur irrespective of PD-L1 expression. Although most trials have analyzed the data based on a prespecified cut-off for PD-L1 on IHC, the results do not consistently show improved responses with higher PD-L1 expression an observation that may not be surprising given that PD-L1 assays are not uniform across clinical trials – neither in the assays utilized nor in the scoring of results. While pembrolizumab and nivolumab clinical trials have used the DAKO assays, Ventana assays have been used with durvalumab and atezolizumab. In the pembrolizumab and nivolumab trials PD-L1 tumor cell staining has been used, whereas the IM vigor trials uses PD-L1 immune cell staining. The cut-offs for PD-L1 staining are also different (Tables 2–4). Intratinal variability in IHC staining may be responsible for variability in the observed responses with different immune checkpoint inhibitors. Other factors worth considering in using PD-L1 as a standalone biomarker for response to immunotherapy is the intratumoral heterogeneity in its expression and its dynamic nature in space and time during the course of disease [103,104]. Taken together there is an unmet need for an ideal biomarker that predicts response to the immune checkpoint inhibitors.

**Tumor mutational burden**

Genome wide analysis has revealed certain tumor specific features that could be used effectively as a biomarker to predict response to therapy with an immune checkpoint inhibitor. Higher frequency of gene mutations, denoted as tumor mutational burden (TMB) is defined as the total number of mutations per coding area of a tumor genome and a higher number of mutations increase the chances of generating neo-tumor-antigens recognized by the host immune system as immunogenic neoantigens [105–107]. TMB is quantified as the number of coding somatic mutations per megabase (MB) of DNA [106]. Tumors with high TMB have been demonstrated to have a microenvironment rich in immune cells and Th1-associated cytokines [108]. Recent data suggests immune checkpoint inhibitors to be more active in tumors with high mutation rates. Emerging data from The Cancer Genome Atlas indicated that urothelial cancers harbored the third highest mutation rate.
DNA mismatch repair and microsatellite instability

Mutations in the mismatch repair (MMR) genes result in formation of microsatellites. These are short, repetitive sequence of DNA, where DNA polymerases are particularly known to be error prone [112]. This leads to DNA damage by single base pair insertions and deletions (indels). MMR proteins are responsible for nucleotide base excision and repair. These proteins are coded by 4 MMR genes namely, MLH1, MLH2, MLH6, and PMS2. A mutation in these genes leads to deficiency in MMR. A MSI-H is a hallmark of deficient mismatch repair or dMMR. Defects in the DNA repair machinery leads to a tremendous increase in rate of mutations [113]. In MSI-H cancers the accumulation of errors in microsatellites results in the so called “mutator phenotype,” an array of abnormal peptides that might represent a pool of tumor specific antigens that render MSI-H tumors inherently more detectable by the host immune system [114]. Put together these factors are predictive of a response to the immune checkpoint blockade tumors that are MSI-H.

Urologic malignancies also display a deficient MMR signature as evidenced by their increased incidence in the patients with Lynch syndrome (LS). Upper tract urothelial carcinoma is the third most common malignancy in LS with an incidence of 1%-5% in this setting. In a study including 115 patients with upper tract urothelial carcinoma 13.9% met the criteria for possible LS and 5.2% had confirmed LS using the Amsterdam II and MSI-IHC criteria for LS. [115] Epidemiologic data and molecular characterization suggest urothelial carcinoma (1%-20%) and prostate cancer (2- to 5-fold increase in incidence) as unrecognized components of Lynch syndrome [116]. Using the MSI-calling software, MANTIS, researchers from Ohio State University, performed whole-exome sequencing on 11,139 tumor-normal pairs across 39 cancer types. Based on their data, MSI-H was detected in 0.49% of the bladder carcinomas, 1.47% of clear cell RCC, 0.6% of prostatic adenocarcinoma, and none of the testicular tumors [117]. These numbers are small in comparison to other cancers such as colonic adenocarcinoma where 19% tumors are MSI-H. Nevertheless, this small subset of patients could very well be the ones which are most responsive to the immune checkpoint inhibitors. Indeed Le et al demonstrated durable responses with pembrolizumab in metastatic cancers with dMMR across a variety of solid malignancies [118,119].

The Cancer Genome Atlas subtypes of the urothelial carcinoma

Based on the gene expression profiling, urothelial carcinomas are classified into basal and luminal subtypes. Basal type tumor cells have higher PD-L1 expression on the tumor cells and immune cells [109]. The Cancer Genome Atlas subtypes have been associated with prognostic differences in the survival and responses to immunotherapy. In the IMvigor 210 trial, the luminal cluster II subtype had a statistically significant higher response rate compared to luminal cluster I, basal cluster I, and basal cluster II subtypes [51,120]. In the CheckMate 275 trial with nivolumab; however, improved responses were seen in basal I subtype followed by luminal II subtypes [54]. Thus, there is a variation in responses between the cluster subtypes across the clinical trials.

Immune gene expression profiling

Tumor immunity results from a complex interplay between tumor cells and immune cells in the tumor microenvironment. A comprehensive immune gene expression profiling of these cells along with the chemokine and cytokine milieu may truly represent the ongoing interactions resulting in tumor immunity. In the IMvigor 210 trial in metastatic urothelial carcinoma, a higher CD8+T effector signature (PD-L1 positivity on immune cells by IHC was associated with expression of genes in a CD8+T effector set) correlated with higher complete response rates to atezolizumab. CXCL-9 (P= 0.0057) and CXCL-10 (P= 0.0079) expression, (chemokines representative of the T effector signature) had a higher response to immunotherapy [121]. While in the Checkmate 275 study that examined the activity of nivolumab in metastatic urothelial carcinoma, a 25-gene interferon-gamma (IFN-γ) signature derived from the pretreatment biopsies found a higher objective response rate to nivolumab in tumors with higher values in the IFN-γ gene signature than in those with low values on the IFN-γ expression score [54]. While IFN-γ is known to have favorable effects on antitumor immunity, persistent signaling has been associated with adaptive resistance to checkpoint therapy. One of the most important IFN-γ mediated effects is the increased expression of PD-L1 and PD-L2 [121-123]. Prolonged exposure of cancer cells to IFN-γ signaling leads to expression of a number of ligands for T cell inhibition, which in turn leads to resistance to immune checkpoint inhibitors independent of the PD-1/PD-L1-pathway [124]. TGF-β signaling in the tumor stroma creates an immunosuppressive phenotype and promotes angiogenesis and metastases. Based on data from the IMvigor210 study, Mariathasan et al showed that increased TGF-β signature - TGF-β ligand, TGFα, and a TGF-β receptor, TGFBR2 - in fibroblasts within the peritumoral stroma was associated with a lack of response and poorer survival to atezolizumab especially in patients where CD8+T-cells were excluded from the tumor parenchyma [125].

Long noncoding RNA

Long noncoding RNAs (lncRNAs) are molecules more than 200 nucleotides in length that do not have the ability to translate into protein [126]. They are transcribed by RNA polymerase II and share a structure similar to mRNA but lack an open reading coding frame [127]. The best documented mechanism by which lncRNAs contribute to tumor immune evasion include the crumpling of the antigen presentation through upregulation of PD-L1 expression and attenuation of T-cell activities [128]. LNMAT1, a long noncoding RNA was found to be over expressed in UC of bladder with lymphnodal metastasis [129].

POLE and POLD mutations

POLE and POLD are essential for proofreading and fidelity in DNA replication. They have mutational frequencies of 2.79% and 1.37% respectively across a variety of cancers including urothelial and prostate cancer. Their germline or somatic mutations can lead to a hypermutated phenotype with high TMB and serve as negative prognostic markers. Mutations in these genes are predictive of survival benefit fromICI therapy, (OS 34 vs 18 m, P= 0.0038)
although much less so when adjusted for MSI status and cancer type (P = 0.047). No significant differences in OS were observed between patients with MSI-H and those patients with POLE/POLD1 mutations who were non-MSI-H [101].

In summary, immune checkpoint inhibitors are gaining widespread usage in first and second-line therapy regimens. Studies that have examined the tumour microenvironment, TMB, MSI status, and expression of immune checkpoints and their ligands have increased our knowledge but it is clear we are still very far from a full understanding of the factors that mediate sensitivity or resistance to immunotherapy.

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