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Immunotherapy for urothelial cancer: from the diagnostic pathologist's point of view

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Inhibitory checkpoint proteins such as programmed cell death-1 (PD-1), programmed cell death ligand-1 (PD-L1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) suppress anti-tumour T-cell responses. Enhancement of such checkpoint proteins is a common immune-evasive strategy in several solid tumours, including urothelial carcinoma (UC).

Immune checkpoint blockade compounds interfere with the tumour-related immune-evasive strategies. Several immunotherapeutic drugs targeting PD-1 and PD-L1 are emerging for the treatment of UC. Some of the drugs have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of advanced or metastatic UC[1-3].

A review paper very recently published in *Expert Opinion On Biological Therapy* gives a comprehensive update on immunotherapy options for UC [4]. The update, as reported in the original paper, can be summarized as follows:

- “Immune-checkpoint inhibitors as a monotherapy are effective for first-line treatment of patients with advanced UC who are ineligible for cis-platinum and also patients who were pre-treated with platinum-based chemotherapy”.
- “The results of trials investigating the efficacy of the combination of immune-checkpoint inhibitors and chemotherapy in the first-line setting are awaited”.
- “It remains unclear whether this combination may become standard first-line treatment in advanced UC.”

Although intravesical Bacillus Calmette–Guérin (BCG) immunotherapy remains the gold standard for non-surgical management of non-muscle-invasive bladder cancer, it is reported to

fail in 40% of patients.[5] Encouraging results have been reported by Balar et al. on the evaluation of the antitumor activity of pembrolizumab in patients with high-risk non-muscle invasive and (BCG)-unresponsive bladder cancer.[6]

PD-L1 TESTING BY IMMUNOHISTOCHEMISTRY

PD-L1 testing was not originally required in UC. FDA and EMA have recently restricted the use of the anti-PD1/PD-L1 drugs Keytruda (Pembrolizumab) and Tecentriq (Atezolizumab)[7]. Both drugs are only indicated as monotherapy in adult patients with locally advanced or metastatic UC, not eligible for cis-platinum-containing chemotherapy and whose tumour is PD-L1 positive by immunohistochemistry (IHC).

PD-L1 IHC testing is now required in the selection of patients with UC. There are three commercially available PD-L1 assays for this purpose (Ventana SP263, Ventana SP142, and Dako/Agilent 22C3), each of them with a corresponding manufacturer's specific algorithm (Figure 1 and Table 1) [8].

Harmonisation of PD-L1 assessment

Pathology laboratories are interested in interchangeable tests to assess PD-L1 expression for several reasons, in particular because Dako and Ventana platforms are not universally available and testing may have to be performed locally. Many harmonisation studies on PD-L1 antibody clones have been performed in non-small-cell lung cancer, where PD-L1 testing has been clinically implemented for many years [9-11]. Data from such studies have shown a high level

of concordance between 22C3, 28.8 and SP263 assays for tumour cell staining, with SP142 as an outlier assay.

Only few harmonisation studies on UC have been reported, all basically focussing on concordance [12-13].

Zajac et al. [14] have just published a study on “Concordance among four commercially available, validated programmed cell death ligand-1 assays in urothelial carcinoma”. They found that good analytical correlation was observed among the VENTANA SP263, PD-L1 IHC 22C3 pharmDx, and PD-L1 IHC 28–8 pharmDx assays for tumor cell (TC) and immune cell (IC) PD-L1 staining. However, concordance between patient PD-L1 status was only achieved for PD-L1 IHC 22C3 pharmDx vs. VENTANA SP263. Differences were observed between patient populations with UC tumors classified as PD-L1 high vs. PD-L1 low/negative using combined positive score (CPS) ≥ 1 , CPS ≥ 10 , IC $\geq 5\%$, and TC/IC $\geq 25\%$. The authors concluded that The VENTANA SP263 and PD-L1 IHC 22C3 pharmDx assays are analytically similar in UC. When the different PD-L1 assays were combined with their specified clinical scoring algorithms, differences were seen in patient classification driven by substantial differences in scoring approaches. Hodgson et al.[15] investigated the concordance of 3 commercial anti-PD-L1 kits (Ventana SP263, Ventana SP142, Dako 22C3) and 1 platform-independent test (Cell Signalling Technologies E1L3N) on 197 UC cases. A high level of concordant PD-L1 among 22C3, SP142 and SP263 have been reported, with 12% of the UC cases showing inconsistent results. Rijnders *et al.* [16] compared 4 commercial anti-PD-L1 kits (Ventana SP263, Ventana SP142, Dako 22C3 and Dako 28.8) and 1 platform-independent test

(Cell Signalling Technologies E1L3N) on 139 muscle-invasive UC cases. An agreement of 80-90% between the 4 commercial anti-PD-L1 kits has been addressed. Another study also investigated the comparability of 4 commercial anti-PD-L1 kits and reported an outlying staining behaviour of the SP142 for tumour cell based PD-L1 scores. Furthermore, this study has highlighted clinical relevance between the currently used scoring algorithms and cut-offs [17].

Major limitations in such studies are the use of tissue micro-arrays (hampering the evaluation of tumour heterogeneity), the very low numbers of evaluating pathologists (limiting the inter-observer variability), the absence of assay concordance and algorithm concordance data, the lack of prospective validation and response data [18].

Nonetheless, PD-L1 expression is modulated at the genetic and epigenetic level and can vary and may potentially increase during PD-1-PD-L1 blockade in some patients. Several factors are responsible of its IHC expression: the upregulation of mRNA expression can be due to an amplification of the CD274 gene encoding PD-L1, methylation of CD274 gene leads to a suppression of its transcription. The transcription is also activated in response to different signalling pathways and transcription factors such as HIF1- α , Myc, Stats, NF- κ B, and AP-1, which are controlled by other interconnected pathways (PI3K/AKT/MTOR, Ras/Raf/MEK/ERK, IFN- γ /JAKs). [19]

UC VARIANTS

The 2016 World Health Organization classification has refined criteria of pathologic features defining several variants of UC [20]. Patients whose tumour is predominantly characterized by variant histologies (i.e., >50% of a certain component within the tumour specimen) usually display an aggressive clinical course as well as a poor response to conventional chemotherapy, except for the small-cell neuroendocrine carcinoma with distinct chemotherapy sensitivity [21-23].

Nearly two-thirds of the variant squamous cell carcinomas of UC exhibit PD-L1 protein expression, in contrast to low expression in conventional UC (20%). Such data suggest that this variant is an attractive target for PD-1/PD-L1-based immunotherapy. In addition, its association with basal-like molecular subtypes (See below in the section “UC subtypes”) and the high co-occurrence rate for potentially targetable oncogenic alterations (phosphoinositide-3-kinase, catalytic, alpha polypeptide, PIK3CA, and Epidermal growth factor receptor, EGFR) raise the possibility of combinatorial and personalized therapeutic approaches for such aggressive tumours in neoadjuvant, adjuvant, and/or metastatic settings [24-25].

In a recent study by Necchi et al neoadjuvant pembrolizumab was used in patients with the predominant variant histology squamous cell carcinoma or with lymphoepithelioma-like (LEL) features. Six of the seven patients (86%) with squamous cell carcinoma had downstaging to $pT \leq 1$, with one pT0 and two of three LEL variants had a pT0 response. On the contrary, no pathological response was observed in any of the other UC with variant histology. [26-27].

Squamous, sarcomatoid, and plasmacytoid variants tend to have greater intra-tumoral T cell CD3 infiltration than conventional UC. Interestingly, sarcomatoid variant has been reported to show a significantly higher PD-L1 score as compared to the nested, glandular, and conventional UC, a finding also reported in lung and kidney cancer. [28-30]

Furthermore, the retrospective analysis conducted by Necchi et al. reported that squamous cell carcinoma harbours the highest frequency of CD274 amplification (gene encoding for PD-L1) and a significantly higher median TMB than adenocarcinoma ($p < 0.001$).[31]

UC SUBTYPES AND PD-L1 expression

UC molecular subtypes

UCs could be basically assigned to two molecular subtypes, such as luminal and basal [32]. Both subtypes may have different sensitivity to current chemotherapy and/or immunotherapy. Exploratory analyses in many trials correlated retrospectively The Cancer Genome Atlas (TCGA) urothelial cancer subtype with response to PD-1/PD-L1 based immunotherapy [33].

The technology needed for comprehensive molecular analyses, such as high-throughput genome analysis, is expensive. It is not applicable for routine diagnostics. It has been suggested that the immunohistochemical expression of only two markers, luminal (Cytokeratin 20) and basal (Cytokeratin 5/6) should suffice to identify the molecular subtypes of UC in an accurate

manner, thus representing a molecular subtyping potentially applicable in the daily practice in all pathology laboratories [34-35].

According to results presented in the IMvigor 210, a phase II trial of Atezolizumab in platinum-treated metastatic or locally advanced UC [36], as well as in other retrospective studies, PD-L1 expression on tumour infiltrating immune cells appeared to be highly expressed in the basal vs. the luminal subtype (i.e., 60% vs. 23%). High PD-L1 expression in tumour cells was reported in the basal subtype (i.e., 39% in basal vs. 4% in luminal). This did not correlate with an objective response rates [37-38].

Similar results have been obtained in the latest taxonomy (i.e., BOLD classification) published by Tan *et al.* [39]. Of the six molecular subtypes with different overall survival and molecular patterns, high infiltration of immune cells was present in the mesenchymal-like (MES) and in the squamous cell carcinoma subtypes (SCC). Both molecular subtypes belong to the basal type. They are represented by cluster III and IV of the TCGA classification [33]. Pathway analyses revealed that PD-1 and CTLA-4 expression were enriched in the MES and SCC subtypes. The immune cell infiltrate between subtypes was different. The luminal-like has greater T-cell CD8⁺ infiltration, while MES and SCC subtypes have higher infiltration of tumour-associated macrophage M2 [39].

There are disadvantages with molecular subtyping, in particular with TCGA subtyping: This technique is difficult to standardize, patients treated with immunotherapy are often restricted to small cohorts and the status of the immune microenvironment is not assessed [18].

Tumor infiltrating immune cells and transforming growth factor β

It has been shown that a gene set associated with CD8⁺ T-effector cells is highly correlated with immune cell infiltrate, with complete response to atezolizumab. In the IMvigor210 study [36], a higher response rate was seen in the luminal (cluster II – TGCA[33], similar to HER2 (human epidermal growth factor receptor 2)-like in the BOLD classification [39]) subtype, characterized by the presence of activated T effector cells. Basal clusters III/IV also showed high PD-L1 immune-cell expression as well as CD8⁺ effector genes, including a high PD-L1 expression on tumour cells. One of the explanations why basal clusters presented with response rates different from luminal clusters might be the presence of immunosuppressive factors. This prevents the activation of the PD-L1/PD-1 pathway by inhibiting effective T-cell activation.

One of these immunosuppressive factors linked with lack of response is a signature of transforming growth factor β (TGF- β) signalling in fibroblasts, a cytokine connected with several pro-tumorigenic effects such as promoting immunosuppression, angiogenesis, fibroblast activation and metastasis. Mariathasan *et al.* [40] showed that the inhibition of TGF- β receptor signalling by anti-TGF- β targeted antibodies increased the ability of Atezolizumab, an anti-PD-L1 targeted agent, to increase anti-tumour immunity by increasing the amounts of CD8⁺ T-cells in the tumour bed and by reprogramming peritumoral stromal fibroblasts.

TUMOR MUTATIONAL BURDEN

An important role to the efficiency of immunotherapy is tumour-specific antigens, i.e., neoantigens. They distinguish tumour cells from normal cells and can stimulate a tumour-specific immune response. The tumours which belong the luminal cluster II-HER2-like have a high neoantigen load and tumour mutational burden (TMB), this being linked with a durable immune checkpoint inhibition response. At the same time, HER2-like demonstrates lower hypoxia signalling, elevated DNA-replication/cell-cycle signalling, high PPAR γ (Peroxisome proliferator-activated receptor gamma) and MRE11 (Double-strand break repair protein MRE11) expression, all linked to good outcome when bladder-preserving trimodal treatment (maximal transurethral resection, radiotherapy, and chemotherapy or immunotherapy) is applied [41].

There are disadvantages with TMB. For instance, it is difficult to standardize between sequencing assays, the relationship between TMB and neoantigen burden is still poorly defined and it does not assess the status of immune microenvironment [18] (Figure 2).

IMMUNE GENE EXPRESSION PROFILING

A difficulty with PD-L1 status as a predictive biomarker is represented by the fact that subjective scoring in IHC tissue sections gives information regarding only a single factor in the microenvironment of the tumour. An advantage of immune gene expression profiling is that RNA can be evaluated and quantified from multiple cell types within a single specimen, and

therefore be more representative for the entire tumor microenvironment. In addition to that, immune expression profiling has the potential to precisely quantify the inflammatory status of a certain tumour by evaluating cytokines, chemokines, as well as cell surface proteins. They can better approximate a “hot tumour” than PD-L1 expression alone [42-43]. There are a few investigations who have dealt with success with immune gene expression profiling by using tissues from different tumour types [44].

A subset of 18 specific genes, selected from 680 different genes analysed with a Nanostring technique, is evaluated prospectively in 3 Phase III trials with pembrolizumab (NCT02628067 [45], NCT02559687 [46], and NCT02564263 [47]). The utility of Nanostring-based gene expression signatures to predict response to immunotherapy depends on the results of such studies. If successful, the information should provide treatment decisions in UC.

There are some disadvantages with immune gene expression profiling. In particular, the lack in standardization of the commercially available gene panels [18].

FUTURE DIRECTIONS

Although PD-L1 IHC testing is required in selected UC patient populations, it is becoming clear that the predictive value of PD-L1 alone for immune checkpoint blockade selection in advanced UC might not be enough. In the last decades, translational research using human samples and mouse models has investigated multiple mechanisms that are involved in the

antitumor immune response. In bladder cancer, histotype variants and molecular subtypes are associated with different sensitivity to immunotherapy/chemotherapy. On the other side, the host-specific parameters, as type of immune infiltrate, activation and infiltration levels, immune gene expression profiling, are important factors that need to be assessed. The future prospective is to incorporate all these factors in a predictive model of response based on currently available clinical and translational data, that can be applied in the clinical practice. [48].

CONCLUSIONS

Numerous clinical and molecular biomarkers that might predict immune response are under examination, including PD-L1 expression in tumor cells and immune cells, tumor mutational burden, molecular subtypes defined by gene expression profiling signatures, and the host immune system activation. All these potential biomarkers are subjects to technical issue such as reproducibility, different score algorithms, accuracy, test reliability, dynamic changes, tumor heterogeneity, sampling variability, and lack of standardization. Though, prospective validation and clinical utilization of putative biomarkers face challenges.

As reported by Lovitch and Rodig [49], “As immunotherapy gains in complexity and is used in combination with agents that target oncogenic, intracellular signaling pathways, diagnostic pathologists will play an increasingly important part in identifying and quantifying cellular and molecular biomarkers in tissue samples that reflect the nature and magnitude of the antitumor immune response”.

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Declaration of Interests

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Table 1. Diagnostic PD-L1 expression assay evaluation for bladder cancer

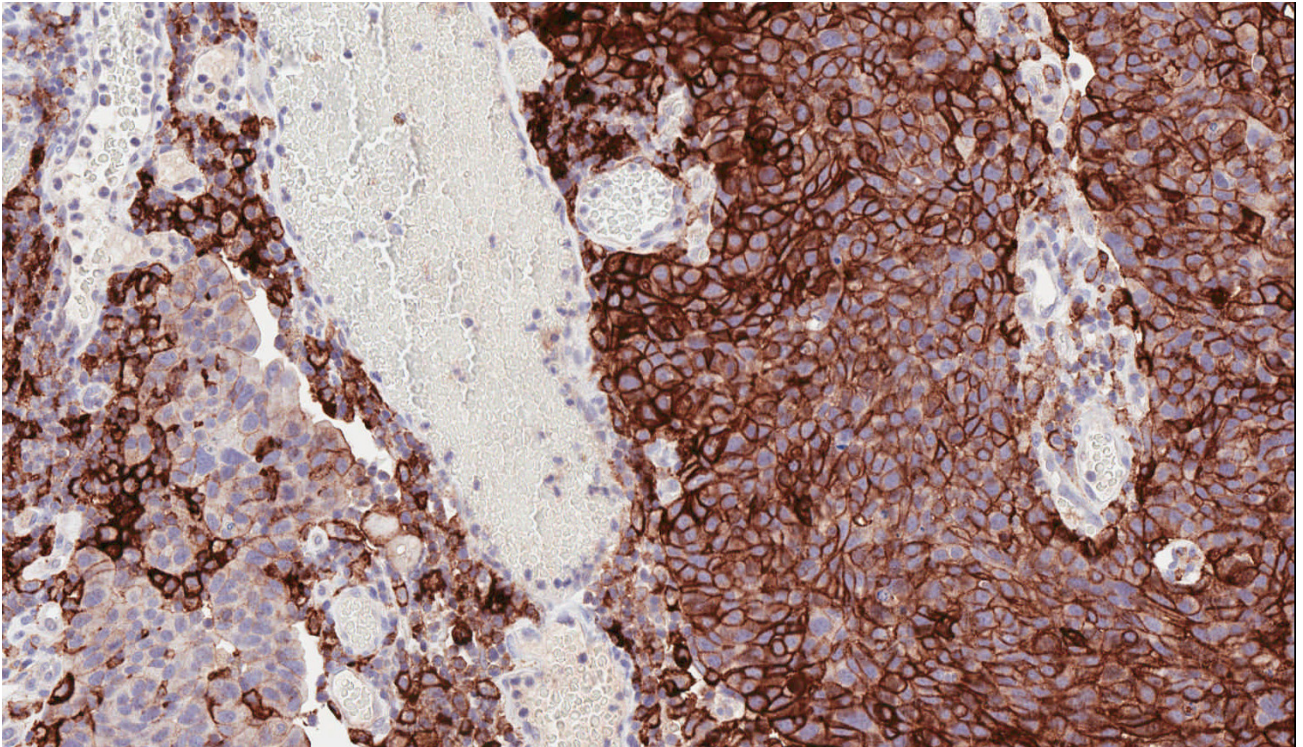
Companion diagnostic assay (Manufacturer)	PD- protein expression evaluation (Algorithm)	IO-Drug Trials
SP263 (Ventana)	assessed with the immune cells (IC)/tumour cells (TC) algorithm and considered positive if $\geq 25\%$ TCs or ICs are immunoreactive ⁵	Durvalumab NCT01693562
SP142 (Ventana)	staining is scored with the IC score and recorded as positive when the positive ICs represented $\geq 5\%$ of the TC area ⁶	Atezolizumab IMvigor 210 trial IMvigor 211 ABACUS trial
22C3 (Dako)	assessed with the combined positive score (CPS) and considered positive when the CPS-score is $\geq 10\%$ ⁷ . CPS is calculated by combining the positive TC and IC staining and expressing it relative to the total TCs ⁷	Pembrolizumab KEYNOTE-052 KEYNOTE-045 PURE-01 trial KEYNOTE-057

Figure legends

Figure 1: PD-L1 expression in invasive high grade urothelial carcinoma. Strong and diffuse membranous expression is seen in the epithelial cells (Right) whereas it is mostly cytoplasmic in the tumor infiltrating immune cells (left) (PD-L1 IHC 22C3 pharmDx by Dako/Agilent)

Figure 2. Complex interplay of chemokines and cytokines classify the inflammatory state of the tumor microenvironment. Interferon-g (IFN-g) released by activated T cells and NK cells activates STAT1, IDO-1 (indolamine oxygenase I) and CMKLR1 in dendritic cells and macrophages (1). STAT-1 mediated signaling and additional pathways produce the chemokines CCL5 and CXCL9 (2). This recruits additional T cells into the tumor microenvironment through CXCR6. IFN-g stimulates the expression of HLA molecules and proteasome components including PSMB10 (3). Finally, IFN-g upregulates a number of immune checkpoint molecules including PD-L1, PD-L2, TIGIT, LAG-3, and B7-H3 on T cells (4). Reproduced under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>) – Figure 4a Ref [18]. J Immunother Cancer. 2017; 5: 94.

Figure 1



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Figure 2

