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THE PD1/PD-L1 AXIS IN THE PATHOGENESIS OF UROTHELIAL BLADDER CANCER & EVALUATING ITS POTENTIAL AS A THERAPEUTIC TARGET

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Urothelial Bladder Cancer (UBC) has a global prevalence of 2.7 million [1], with around 429,000 new cases diagnosed worldwide in 2014, with 165,000 patients deaths each year [2]. At presentation over three quarters of UBCs will be confined to the mucosa or lamina propria (Non-Muscle-Invasive Bladder Cancer, NMIBC: stages Ta/T1/Tis), whereas the remainder are more invasive penetrating the underlying detrusor muscle and beyond with an ability to metastasise (Muscle-Invasive Bladder Cancer, MIBC: stages T2-T4) [3]. Of those patients with NMIBC, around 40% eventually progress to MIBC, of whom 20% will die from metastatic disease [4]. Advanced metastatic MIBC has a very poor 5-year survival rate of around 5%, compared to 80-90% for NMIBC [4]. Unfortunately, an absence of 'tailored' therapies for metastatic UBC is one contributing factor to the lack of significant improvement in outcomes for the last 30 years, with median survival rates remaining at 15 months. An unmet need exists for the development of efficacious and well tolerated therapies for metastatic UBC to improve the guarded prognosis and dismal survival still seen in these patients.

In 2011, Hanahan and Weinberg implicated the role of immune evasion in cancers to allow tumourigenic progression [5]. The developing field of immunotherapeutics has since aimed to target this notion, utilising monoclonal antibody therapy to block T cell immunological checkpoints in the management of UBC. In particular, members of the Programmed Cell Death Protein 1 (PD-1) / Programmed Death Ligand 1 (PD-L1) signalling axis have been promising targets, with multiple studies demonstrating that higher levels of PD-L1 expression on tumour cells may facilitate tumour progression and promote tumour invasiveness and metastatic progression [6-8]. These results have led to breakthrough

therapy designation by the FDA for the anti-PD-L1 antibody MPDL3280A in metastatic UBC [9].

In this paper we discuss the mechanism for immune-evasion by UBC cells using the PD-1/PD-L1 signalling axis; we also evaluate the use of anti-PD-L1 therapy in metastatic UBC, and its viability as a future clinical treatment.

The PD-1/PD-L1 signalling axis impedes an effector T-lymphocyte response towards tumour cells

The PD1 protein is an inhibitory transmembrane receptor expressed primarily on the surface of CD4+ and CD8+ T lymphocytes, but is also present on B lymphocytes and NK cells. Upon interaction with its cognate ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC), it plays a fundamental role in regulating T-cell proliferation and suppressing elevated cytotoxic T-cell activity in peripheral tissues upon infection, thus acting as a tolerance mechanism for the prevention of autoimmunity [10]. As such, the PD-1/PD-L1 pathway has been recognised within the tumour microenvironment as a method of immune-evasion. The upregulation of PD-L1 expression on the surface of UBC cells causes subsequent evasion of a T-cell effector response which aids tumour invasiveness. Furthermore, increased expression of PD-L1 is associated with a higher tumour grade and poorer survival rates [6-8].

Currently, two models exist implicating the method of PD-L1 upregulation on tumour cells: innate immune resistance and adaptive immune resistance [11]. The innate immune resistance mechanism is activated upon constitutive oncogenic signalling inducing PD-L1 expression on tumour cell surface. The adaptive immune resistance mechanism occurs in

response to a previously mounted antitumor immune response due to cytokine production. Innate immune resistance relies upon constitutive oncogenic signalling in the tumour cell which drives the upregulation of PD-L1 on tumour cells. Parsa *et al* first demonstrated PD-L1 expression on tumour cells upon constitutive activation of the AKT signalling pathway, and more specifically this expression was enhanced as a result of a deletion or silencing of the tumour suppressor, *PTEN* [12]. However, this was demonstrated only *in vitro* using a glioma cell line, thus results cannot be directly applied to UBC. Despite this, loss of *PTEN* is a key carcinogenic process demonstrated in MIBC cell lines. Mutated *PTEN* inactivates mTOR leading to dysregulated AKT activation which ultimately upregulates PD-L1 in these cells [13]. In contrast, in the adaptive mechanism, PD-L1 expression occurs only in response to a previously propagated antitumour immune response. This relies strongly on PD-L1 expression occurring in response to cytokine stimulation; in particular, interferon gamma (IFN-γ) production from the surrounding epithelial and stromal microenvironment [8]. Liu *et al* demonstrated that the IFN-γ and TLR ligands activate PD-L1 expression via a common signalling pathway involving Myd88/MEK/ERK/STAT1 [14]. Furthermore, synthetically blocking ERK signalling via an ERK inhibitor leads to inhibition of PD-L1 expression and also functionally demonstrates increased Cytotoxic Lymphocyte (CTL) killing of UBC cells, reiterating the importance of this pathway in activating the PD-1/PD-L1 axis [15]. Ultimately, the effector functions of PD-1/PD-L1 interaction are mediated through dephosphorylation and inhibition of hundreds of interconnecting downstream signalling kinase pathways through recruitment of SHP250 phosphatase [16]. This inhibits signalling events downstream of the TCR, including PI3K/Akt activation, limiting T-cell proliferation, promoting the differentiation of CD4+ T cells into Fox P3+ Treg cells and governing tumour

cells' resistance to cytotoxic T lymphocyte attack [11]. The individual molecular details of these signalling pathways are beyond the scope of this literature review.

PD-L1 expression levels are associated with increased tumour staging and aggressiveness

The expression of PD-L1 on UBC cells facilitates evasion of immunological T-cell targeting, resulting in tumour propagation. It has therefore been postulated that through quantifying the amount of PD-L1 within a tumour sample, it may predict the extent of tumour aggressiveness.

Nakanishki first analysed and identified the percentage of PD-L1 expression (B7-H1) on UBC cells, showing a statistically significant association with tumour grade (p = 0.01) and stage (p = 0.031) [6]. Grade 1 tumours demonstrated 8.12% PD-L1 expression (mean: 8.12 SD+/- 0.05) compared to 26.1% expression (mean: 25.6 SD +/- 0.09) in grade 3 tumours. Additionally, Ta tumours and CIS demonstrated 16.4% PD-L1 expression (mean: 16.4 SD+/- 0.12) compared to 34.7 % (mean: 34.7 SD+/- 0.13) in T4 tumours**.** Inman and colleagues confirmed this finding in a cohort of 280 UBC samples evaluated for PD-L1 expression by immunohistochemistry (IHC), demonstrating that stage progression was strongly associated with the extent of PD-L1 expression [7]. They showed that 28% of tumour specimens stained positive for PD-L1 (defined as plasma membrane staining of greater than 1% of tumour cells), of which 7% of pTa tumours had PD-L1 positivity in comparison to 30% of T3/4 tumours. Univariate statistical modelling showed that PD-L1 expression had an association with higher grade tumours (OR 2.4 [95% CI: 1.2-4.72]) (p= 0.09). However, there are limitations to this study. Firstly, it has been demonstrated that there is a disparity in PD-L1 expression between fresh resected material and historic paraffin-embedded samples, and

this could account for the lack of PD-L1 positive tumours in this study [8]. Furthermore retrospective studies are prone to selection bias, and combined with discrepancies in defining PD-L1 positivity between studies (>5% vs >1%), this may not be truly representative of PD-L1 expression in UBC.

Bellmunt *et al* recently demonstrated a lack of association of PD-L1 with tumour stage or outcome in NMIBC and MIBC (PD-L1 expression of 41.8% vs 30%, respectively, p=0.53]).[9] It is important to note that due to the focal nature of PD-L1 expression within tumours combined with considerable intratumour heterogeneity in UBC, the three different sampling cores utilised in the study may not have been adequate.

A meta-analysis by Zhang in 2015, which examined the expression profiles of PD-L1 in a range of epithelial cancers including UBC, demonstrated that UBCs did not show a significant increase in overall survival in tumours which were PD-L1 negative (HR 1.06, 95% CI 0.71–1.58, p=0.761) [16]. Conversely, comparing PD-L1 negative tumours with PD-L1 positive tumours showed increased overall survival in all of the epithelial cancers in the meta-analysis (HR 1.81, 95% CI 1.33-2.46, p<0.001). Overall, they concluded that there was an 81% increase in risk for all time mortality associated with PD-L1 positivity. By combining PD-L1 expression and outcome data from this group of histologically and morphologically similar tumour types, the authors have potentially identified an overall significant effect between PD-L1 expression and tumour stage and survival. However, the current lack of available data in UBC and relatively small non-representative samples may be masking a genuine association between PD-L1 expression and important clinical variables, limiting the identification of statistically viable relationships. Furthermore, the lack of prospective data

and the lack of standardised antibodies and cut-offs implies that a valid conclusion cannot yet be made.

Anti-PDL1 therapy can enhance intratumoural immune responses leading to tumour regression in metastatic UBC

Antibody targeting of PD-L1 expressed on tumour cells primarily acts through inhibiting molecular binding to PD-1, thus mobilising tumour-specific CTLs to mediate destruction of PD-L1 expressing tumour cells. The increased expression of PD-L1 on tumour cells in a diverse range of cancers has generated an important rationale for the capacity of monoclonal antibody blockade of this pathway to enhance intratumoural immune responses, and prevent invasion and therefore progression [11].

Iwai et al functionally demonstrated the anti-tumorigenic effects of inhibiting the PD-1/PD-L1 axis. Using an *in vivo* modelling experiment with PD-1 knockout mice and subcutaneous injection of a PD-L1 positive J558L myeloma cell line, they showed that there was complete suppression of tumour growth when compared to mice expressing normal PD-1 [17]. Tumour volumes reached up to 6800mm3 in the PD-1 +/+ mice 18 days post inoculation, compared to completely suppressed growth in the knockout mice.

In the clinical setting, Brahmer demonstrated the significant efficacy associated with anti-PD-L1 therapy in advanced metastatic cancer. In a group of 160 patients with renal cell, colorectal, ovarian and pancreatic cancers, they demonstrated durable tumour regression effects in all of the cancer subgroups with objective responses in 6 to 17% of patients [10]. In addition, there was prolonged stabilisation of disease at 24 weeks in 12-41% patients. These results demonstrate the potentially considerable clinical benefits of anti-PD-L1 therapy in cancer patients with a poor prognosis. The study also highlighted the low toxicity

associated with anti-PD-L1 therapy with 91% of drug-related adverse events being low grade, of which fatigue and loss of appetite were most common; treatment-related grade 3 or 4 events were observed in 19 out of 207 patients. Thus, the therapy appears to be well-tolerated in advanced metastatic cancer.

The recent results of an expanded phase I trial using MPDL3280A (anti-PD-L1) in a heavily pre-treated advanced UBC population similarly showed promising clinical outcomes [9]. In 67 patients after 6 weeks of follow-up, 43% of patients with positive PD-L1 expression achieved an objective response compared to 11% of patients with negative PD-L1 expression. In addition, 7% of the positive PD-L1 patients achieved a complete response at 6 weeks after treatment, reinforcing the efficacious effect of anti-PD-L1 therapy in patients with advanced disease who have previously failed chemotherapy. However, the response to MPDL3280A was more associated with PDL-1 expression in tumour infiltrating immune cells (TIICs) ($p<0.026$) than expression in UBC tumour cells ($p<0.93$). The role of TIICs and their PD-L1 expression may thus provide an important mechanism for determining the likelihood of response to anti-PD-L1 therapy, and may be utilised as a predictive biomarker. However, further work is required since TIICs were characterised by morphology only; we suggest greater molecular characterisation is warranted through co-immunostaining for CD11b, Granulocyte receptor 1 and CD3 to definitively confirm their origin. Furthermore, the small pool of PD-L1 negative patients who responded to therapy raises a concern for the implications of excluding PD-L1 negative patients and henceforth excluding potential responders to therapy. PD-L1 positive and negative groups were also not well balanced (7 and 58 patients, respectively) which questions the overall power of this study.

Future clinical implications for anti-PD-L1 therapy

Deng *et al* recently reported the effectiveness of anti-PD-L1 used as a combinational therapy with high dose ionising radiotherapy (IR). They noted that PD-L1 expression was induced in tumour cells after IR and so could represent a potential mechanism for immune evasion (and resulting in high tumour relapse rates after treatment)[18]. Combining anti PD-L1 with IR showed a statistically significant restriction in tumour growth compared to IR alone after 31 days: IR monotherapy tumour size: 402.8 ± 76.73 mm vs. IR + anti PD-L1 tumour size: 25.59 ± 10.26 mm) (p = 0.0002). These results demonstrate a 16-fold difference in tumour size despite similar baseline growth characteristics prior to treatment. IR appeared to slow tumour progression whereas the combinational therapy of IR and anti-PD-L1 dramatically restricted tumour growth and enhanced the effects of IR. Additionally, anti-PD-L1 therapy alone showed modest tumour size of 587.3 ± 169.1 mm, although less effective than IR as a monotherapy. This indicates the efficacy and clinical benefit of this treatment is best suited as combinational therapy and may require IR to dynamically enhance the levels of PD-L1 expression to a clinically efficacious threshold which then can be inhibited, thus downregulating the numerous associated signalling pathways which drive tumour progression. However, these results were trialled using a mammillary carcinoma cell line, which may not be fully characteristic of the cytokine milieu and behaviour of UBC cells. Despite this, radiotherapy is a common treatment for MIBC and so further investigation is warranted to determine whether PD-L1 expression is induced in UBC after IR and whether there are potential therapeutic benefits of combinatorial anti-PD-L1 therapy.

We have discussed the importance of anti PD-L1 therapy in targeting advanced metastatic MIBC. However, there may also be utility in the NMIBC setting to prevent tumour progression. Inman first characterised Carcinoma in situ (CIS) as having the highest levels of PD-L1 expression, with a greater than 10% increase in PD-L1 positivity than late stage pT3/4 UBC tumour cells[7]. This is of paramount importance as approximately 50% of CIS patients experience stage progression. Intravesical BCG immunotherapy combined with aggressive endoscopic removal does not, unfortunately, confer life-long immunity, with BCG failure occurring on average after 5 years. It appears therefore, that BCG's initial immunosuppressive mechanism against CIS is overcome by enhanced intratumoural PD-L1 expression and infiltrating lymphocytes.

Although 45% of CIS lesions are PD-L1 positive, there is a low median intratumoural expression of just 5% of cells [7]. However, CIS patients who failed BCG had a 20-fold increase in PD-L1 expression. This highlights the importance of the PD1/PD-L1 axis in driving tumour progression and in developing an adaptive resistance mechanism to BCG therapy, and the potential for anti-PD1/PD-L1/BCG combination therapy. There is an urgent need for further studies to elucidate a mechanistic involvement of PD-L1 in BCG failure, and to explore whether this can be exploited.

Conclusion

Appreciating the molecular mechanisms underpinning PD-1/PD-L1 signalling in UBC is imperative in highlighting the rationale behind its use in immunotherapy. Empirical evidence in a range of cancers suggests PD-L1 expression may be induced upon a complex interplay between constitutive oncogenic signalling and inflammatory cytokine stimulation from the

tumour microenvironment [13-15]. This background is mechanistically important in characterising the behaviour of UBC cells which may be responsible for propagating tumour invasiveness and treatment resistance [19]. Henceforth, it identifies a new, pharmacologically tailored therapeutic target to be utilised in patients who are already chemo/radiotherapy resistant and are in the late stages of aggressive or metastatic MIBC.

We have also discussed the implications of PD-L1 status and whether it negatively associates with reduced survival and increased tumour aggressiveness. There is a current discordance in medical literature about whether PD-L1 expression is responsible, with statistically significant data existing for both viewpoints. The current available literature in bladder cancer is primitive and has too many confounding factors. We suggest that further observational studies are required using larger sample populations with a consistent methodology for assessing PD-L1 status. This will involve the development of a standardised PD-L1 antibody for IHC, agreeing a PD-L1 expression cut-off value, and assessing whole tumour sections (instead of tumour core biopsy samples) to account for the focal nature of PD-L1 expression patterns.

Furthermore, the use of anti PD-L1 therapy has identified that a small responsive pool of PD-L1 negative patients (15%) achieved objective responses to therapy [9]. This implies that either there was a failure to identify these patients as PD-L1 positive, or that further underlying mechanisms have resulted in a molecular response to therapy. We hypothesise that infiltrating lymphocytes in the tumour microenvironment which are PD-L1 positive may be the most likely reason behind this observation. This raises concerns as excluding PD-L1 negative patients may exclude potential responders to therapy.

It also re-evaluates the current viewpoint on PD-L1 screening and whether it is even necessary: should pre-treatment PD-L1 assessment be abolished or be expanded to encompass the characterisation of TIL PD-L1 status? Furthermore, current studies have only characterised PD-L1 expression on primary UBCs and do not account for metastases; this should be further evaluated through biopsies of involved lymph nodes and distant metastases. The promising results of Powles' phase I anti-PD-L1 therapy in UBC have resulted in provisional FDA approval [9]. We await the results of the phase II trial which is currently underway.

We also report on the favourable toxicity profile associated with anti-PD-L1 therapy. Considering that the majority of UBC patients are over 70 years old, with an estimated 40% with some level of renal impairment, there is significant importance in ensuring that treatments are well tolerated and can generate objective tumour responses without a trade-off for quality of life. Further longitudinal studies are also warranted to ensure safety and tolerability for patients in the longer term and to ensure no autoimmune toxicity issues arise.

Finally, the future indications for anti-PD-L1 therapy may extend to BCG-resistant NMIBC. This has significant economic incentives due to the patient burden and healthcare costs of NMIBC and the globally limited supply of BCG [20]. We postulate that anti-PD-L1 may circumvent BCG resistance mechanisms and prevent the progression to MIBC and metastatic disease.

In conclusion, anti-PD-L1 is a highly promising therapy in UBC. The wealth of upcoming clinical trials highlights the global scientific enthusiasm surrounding this therapeutic approach which may dramatically improve the lives of UBC patients in the near future.

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