TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Comprehensive Evaluation of Programmed Death-Ligand 1 Expression in Primary and Metastatic Prostate Cancer

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Antibodies targeting the programmed cell death protein 1/programmed death-ligand 1 (PD-L1) interaction have shown clinical activity in multiple cancer types. PD-L1 protein expression is a clinically validated predictive biomarker of response for such therapies. Prior studies evaluating the expression of PD-L1 in primary prostate cancers have reported highly variable rates of PD-L1 positivity. In addition, limited data exist on PD-L1 expression in metastatic castrate-resistant prostate cancer (mCRPC). Here, we determined PD-L1 protein expression by immunohistochemistry using a validated PD-L1-specific antibody (SP263) in a large and representative cohort of primary prostate cancers and prostate cancer metastases. The study included 539 primary prostate cancers comprising 508 acinar adenocarcinomas, 24 prostatic duct adenocarcinomas, 7 small-cell carcinomas, and a total of 57 cases of mCRPC. PD-L1 positivity was low in primary acinar adenocarcinoma, with only 7.7% of cases showing detectable PD-L1 staining. Increased levels of PD-L1 expression were noted in 42.9% of small-cell carcinomas. In mCRPC, 31.6% of cases showed PD-L1-specific immunoreactivity. In conclusion, in this comprehensive evaluation of PD-L1 expression in prostate cancer, PD-L1 expression is rare in primary prostate cancers, but increased rates of PD-L1 positivity were observed in mCRPC. These results will be important for the future clinical development of programmed cell death protein 1/PD-L1-targeting therapies in prostate cancer. (*Am J Pathol* 2018; 188: 1478–1485; <https://doi.org/10.1016/j.ajpath.2018.02.014>)

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Antibodies that block the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) interaction currently are approved by the US Food and Drug Administration for multiple tumor types, including melanoma, non–small-cell lung cancer, kidney cancer, bladder cancer, and several other cancers.^{1,2} The potent clinical activity of these agents supports the notion that up-regulation of PD-L1 expression is a key immune modulatory strategy used by cancer cells and myeloid cells in the stroma to dampen a host antitumor response.^{3,4} In some tumor types (eg, non–small-cell lung cancer), immunohistochemical assessment of PD-L1 is in clinical use as a predictive biomarker,^{1,5,6} although the predictive power of PD-L1 positivity varies widely across agents and histologies. As a broad generality, however, in most tumor types, PD-L1–positive tumors tend to have a higher rate of objective response to PD-1/PD-L1 blockade, and PD-L1 expression remains an important clinically validated predictive biomarker for response.^{7,8} Baseline PD-L1 expression levels vary widely in different tumor types.^{5,6,9,10} In primary prostate cancers, the reported frequency of PD-L1 ranges from 8% to 92%.^{9,11–13} In addition, very limited data exist on the expression of PD-L1 in metastatic prostate cancer. It is important to note that differences in the assessment of PD-L1 expression, including sample processing, antibody selection, and scoring, contribute to the diverse results published in the literature.^{6,14} Therefore, a comprehensive evaluation of PD-L1 expression in both primary and metastatic disease is necessary to guide further clinical development of checkpoint inhibitors in prostate cancer.

In early studies, mCRPC has shown limited responses to PD-1–targeting therapies.¹ However, an interim analysis of an ongoing study of PD-1 inhibition in advanced prostate cancer showed partial responses in 2 of 10 patients.¹⁵ Therefore, the clinical utility of PD-1–targeted therapies remains to be established. Generally, immunotherapies, in particular vaccine-based approaches, have shown activity in advanced prostate cancer. For instance, Sipuleucel-T, an autologous antigen-presenting cell–based immunotherapy, and vaccine-based approaches such as PROSTVAC-VF (Bavarian Nordic, Inc., Morrisville, NC), have shown activity in metastatic castration-resistant prostate cancer (mCRPC), suggesting that immune modulatory approaches hold promise in advanced prostate cancer.^{16–18} Androgen-deprivation therapy remains the mainstay of therapy for advanced metastatic prostate cancer. In particular, with the addition of more potent second-generation anti-androgens such as enzalutamide, and the steroid 17- α -hydroxylase/17,20 lyase inhibitor abiraterone to the armamentarium, the ability to therapeutically interfere with androgen receptor signaling has been greatly expanded.¹⁹ Importantly, androgen ablation has been shown to augment immune responses and exert diverse immune modulatory effects and changes in the tumor microenvironment.^{18,20} Interestingly, in cell line models of CRPC, an increase in PD-L1 expression was noted, suggesting that immune checkpoint molecule expression can change during prostate cancer progression in response to androgen-deprivation therapy.²¹

Here, we evaluated the expression of PD-L1 using a validated, commercially available antibody in primary and metastatic prostate cancer. We show that although the overall positivity in primary prostate cancer is lower than in other solid tumors, some cases with high PD-L1 expression can be identified. Furthermore, we demonstrate that in both biopsy specimens and rapid autopsy specimens of mCRPC, up to 32% of cases show tumor-specific PD-L1 labeling on at least a fraction of tumor cells. Taken together, low-level PD-L1 expression is detectable in primary prostate cancer, with a significantly increased rate of PD-L1 positivity in mCRPC.

Materials and Methods

In Silico Expression Analyses

In silico expression analyses were performed using publicly available data sets. In brief, raw data for GSE35988²² and processed data for Stand Up To Cancer metastatic prostate cancer²³ were obtained from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) and cBioPortal version 1.12.1²⁴ (<http://www.cbioportal.org>), respectively, and processed as previously described.²⁵

Western Blot Analysis

Western blot analysis confirming the specificity of PD-L1 antibodies were performed as described previously.²⁶ Anti-PD-L1 antibody (rabbit monoclonal clone sp263, ref 790-4905; Ventana Medical Systems, Inc., Tucson, AZ) was used at a 1:300 dilution.

Patient Cohorts

PD-L1 expression was examined using three distinct patient cohorts. The first was a collection of primary prostatic tumors from the Johns Hopkins Hospital ($n = 539$) represented on 14 tissue microarrays (TMAs); the second was a collection of core biopsy specimens and surgical resection specimens from men with mCRPC from the Johns Hopkins Hospital ($n = 28$); and the third was a collection of rapid-autopsy tumor samples from men who had died of mCRPC at the University of Michigan ($n = 29$) represented on two TMAs (Supplemental Table S1).²⁷

The Johns Hopkins Hospital mCRPC cohort ($n = 28$) comprised two sample sets. The first set comprised core biopsy specimens of metastatic lesions ($n = 9$) obtained at baseline from mCRPC patients who were enrolled in a prospective clinical trial investigating the oral Hedgehog pathway inhibitor vismodegib.²⁸ The second set of samples ($n = 19$) was obtained prospectively from a biomarker study examining androgen receptor-V7 mRNA and protein detection in men with mCRPC who were about to start a new line of systemic therapy and contained both surgical resection ($n = 10$) and core biopsy ($n = 9$) samples.^{29,30} Details on anatomic location and treatment history for these cases are summarized in

Supplemental Table S2. All patients provided written informed consent before undergoing a biopsy, and both studies were conducted under Johns Hopkins' Institutional Review Board—approved protocols.

Immunohistochemistry

Based on detailed antibody validation and previously published antibody comparisons,^{6,31} the rabbit monoclonal clone SP263 (ref 790-4905; Ventana Medical Systems, Inc.) was used on the entire study cohort. A staining protocol, using SP263, was developed on an automated instrument (BenchMark Ultra; Ventana Medical Systems, Inc.). Staining was performed on 4- to 5- μ m-thick, formalin-fixed, paraffin-embedded tissue sections from various tumors. Human placenta and tonsil tissue were used for optimization and as positive controls. Slides were deparaffinized, hydrated, and heat-induced antigen retrieval was performed with a high pH buffer (CC1; Ventana Medical Systems, Inc., 56 minutes). PD-L1 antibody (rabbit monoclonal clone SP263, ref 790-4905; Ventana Medical Systems, Inc.) was applied for 16 minutes at room temperature and staining was developed with the Opti-View detection system as per the manufacturer's instructions using diaminobenzidine chromogen (Ventana Medical Systems, Inc.). Slides were counterstained with hematoxylin, dehydrated, and coverslipped. For additional selected cases two additional antibodies were used. Tissue controls (placenta and lymphoid tissue) as well as cell line controls (SR and SN12C) were used with all staining batches.

Scoring

Biopsy and resection specimens were evaluated by conventional light microscopy. TMA slides were visualized and annotated using TMAJ software version 3.15 (Tissue Microarray Core Facility, Johns Hopkins University, Baltimore, MD)³² on images acquired on an Aperio Scanscope AT Turbo scanner (Leica Biosystems, Buffalo Grove, IL) at a magnification of $\times 20$. Note that all scoring was performed by two pathologists (G.G., M.C.H.), and all positive cases were reviewed by an expert genitourinary pathologist (A.M.D.). All three pathologists were blinded to the clinical characteristics of the specimens. Adjacent hematoxylin and eosin—stained sections were reviewed in cases of uncertain tumor involvement. Any PD-L1—specific immunoreactivity on malignant cells ($\geq 1\%$) was considered positive.

Microsatellite Instability Analysis

Mismatch repair status was determined in two primary tumor cases that showed high PD-L1 expression using the Microsatellite Instability Analysis System v1.2 (MSI Multiplex Kit; Promega, Madison, WI) and the ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Selected microsatellite sequences that are particularly prone to errors

in the setting of mismatch repair were evaluated as described previously.³³

Results

To establish robust and reproducible controls for the PD-L1 immunolabeling assay integrated copy number and *in silico* expression analyses were performed on previously published data from the NCI-60 cell line panel (Figure 1A).^{24,34} The human lymphoblastic cell line SR showed high levels of PD-L1 (*CD274*) mRNA expression and showed no copy number alteration of the *CD274* locus, in contrast to the human renal cell carcinoma cell line SN12C, which showed copy number loss of the *CD274* locus with associated reduced *CD274* mRNA expression. Several studies have extensively validated commercially available PD-L1—specific antibodies for immunohistochemistry and showed favorable sensitivity and specificity profiles of clone SP263.^{31,35} Based on these prior reports, the specificity of SP263 was characterized further by Western blot analysis of placental tissue and SR and SN12C cell line lysates and identified bands at the predicted molecular weight of PD-L1 in placenta and SR lysates. Corroborating the predictions from *in silico* expression analyses, lysates from SN12C showed no immunoreactivity (Figure 1B). The specificity of the antibody for immunohistochemical analysis of formalin-fixed, paraffin-embedded tissues was confirmed further by immunostaining formalin-fixed, paraffin-embedded cell blocks containing SR and SN12C cells, showing strong membranous immunoreactivity in SR cells and absence of signal in SN12C cells (Figure 1C).

Previous studies evaluating the expression of PD-L1 in primary prostate carcinoma yielded highly variable results.^{11,13,36} To determine the prevalence of PD-L1 expression in primary prostate carcinoma, the expression of PD-L1 was evaluated by immunohistochemistry in a total of 539 primary prostate cancers represented by 6137 tissue cores on 14 TMAs (Table 1). Of 508 primary prostatic adenocarcinomas, 39 (7.7%) showed PD-L1 expression as defined by detectable membranous PD-L1 immunoreactivity in 1% or more of the total cellularity of the lesion. Fourteen cases (2.8%) showed immunoreactivity in 5% or more of cells. Two primary adenocarcinomas showed PD-L1 expression in more than 50% of cancer cells (Figure 2, Supplemental Figures S1 and S2). Given the recently described association between PD-L1 expression and mismatch-repair—deficient carcinomas, the microsatellite instability status was determined in these two lesions and no evidence of microsatellite instability was found (Supplemental Figures S1 and S2; Supplemental Tables S3 and S4). There was a trend for higher PD-L1 expression in higher-grade groups ($P = 0.08$, *t*-test), and in particular high PD-L1 expression ($\geq 5\%$) was associated with Gleason patterns 4 and 5. In addition to conventional adenocarcinoma, the PD-L1 expression also was evaluated in a cohort of prostatic duct adenocarcinomas ($n = 24$) and small-cell carcinomas ($n = 7$), and

Figure 1 Validation of programmed death-ligand 1 (PD-L1) antibody used in the study. **A:** Integrated *in silico* analysis of NCI60 cell lines shows high-level PD-L1 expression in the lymphoblastic cell line SR and absence of PD-L1 expression in the renal cell carcinoma cell line SN12C. **B:** Western blot analysis of placental tissue lysate and SR and SN12C cell line lysates probed with anti-PD-L1 (SP263) shows immunoreactivity only in the predicted molecular weight range of PD-L1. **C:** Formalin-fixed, paraffin-embedded SN12C and SR cells show absence and strong membranous immunoreactivity for PD-L1, respectively. IHC, immunohistochemistry. Original magnification, $\times 20$. WB, western blot.

immunoreactivity was observed in 4 of 24 prostatic duct adenocarcinomas (16.7%) and 3 of 7 (42.9%) small-cell carcinomas (Supplemental Figure S3). In addition to PD-L1 expression detected in neoplastic cells and tumor-associated immune cells, focal PD-L1 positivity was observed frequently

Table 1 Overview of Clinicopathologic Characteristics of PD-L1–Positive and PD-L1–Negative Tumors

		PD-L1 [−]		PD-L1 ⁺	
Tumor type	Cases, <i>n</i>	<i>n</i>	%	<i>n</i>	%
Primary tumors					
Acinar adenocarcinoma	508	469	92.3	39	7.7
Grade group					
1		44	9.4	3	7.7
2		139	29.6	15	38.5
3		124	26.4	4	10.3
4		66	14.1	2	5.1
5		96	20.5	15	38.5
Stage					
T2		179	38.2	16	41.0
T3A		194	41.4	12	30.8
T3B		81	17.3	9	23.1
T4		0	0	1	2.6
Lymph node status					
N0		432	92.1	35	89.7
N1		33	7.0	3	7.7
Prostatic duct adenocarcinoma	24	20	83.3	4	16.7
Small cell carcinoma	7	4	57.1	3	42.9
Distant metastases (mCRPC)					
Rapid autopsies	29	20	69	9	31
Biopsies	28	19	67.9	9	32.1

in benign atrophic glands, in particular, in association with a chronic immune infiltrate, as seen previously (Figure 2).¹²

To evaluate the expression of PD-L1 in the setting of mCRPC, PD-L1 immunoreactivity was determined in mCRPC samples procured by rapid autopsy studies ($n = 29$), surgical resection specimens ($n = 10$), and biopsy specimens ($n = 18$). Note that the majority of patients for whom biopsy or resection specimens were available were treated previously with androgen-deprivation therapy including abiraterone acetate or enzalutamide (Supplemental Table S2). Independent of tumor site, CRPC metastases showed greatly increased incidence of PD-L1 expression, with more than 31% of cases showing PD-L1 positivity ($\geq 1\%$ of tumor cells) and up to 11% showing PD-L1 immunoreactivity in $\geq 5\%$ of tumor cells (Figure 2 and Supplemental Tables S1 and S2). Importantly, despite different sampling methods of tissue acquisition (core biopsy, surgical resection, post-mortem rapid autopsy) the rate of PD-L1 positivity did not vary (Figure 2). In the autopsy cohort, for which multiple metastatic sites from each case were available for analysis, a high level of heterogeneity was observed in PD-L1 expression in different anatomic sites (Supplemental Table S1). In addition, patchy PD-L1 immunostaining of the neoplastic cell compartment was observed in lesions with high PD-L1 expression with strongly labeled focal cancer cell nests located adjacent to PD-L1–negative cells (Supplemental Figure S4). These immunohistochemical *in situ* studies were corroborated by re-analysis from publicly available RNA sequencing studies, showing PD-L1 transcript expression in a subset of mCRPC (Supplemental Figure S5).

Figure 2 Summary of observed programmed death-ligand 1 (PD-L1) immunoreactivity in primary and metastatic prostate cancer. **A:** Bar graph showing relative frequency (%) of lesions with detectable PD-L1 expression. **B:** Representative micrograph of primary prostate carcinoma showing no PD-L1 expression. **C** and **D:** Representative micrograph of metastatic prostate cancer with moderate- and high-level PD-L1 expression. **E:** Focal immunoreactivity in benign atrophic prostate epithelium associated with chronic inflammatory infiltrate. Original magnification, $\times 20$ (**B–E**).

Discussion

Antibody-mediated blockade of the PD-1/PD-L1 axis is effective in multiple solid tumor types.^{1–3} Prior studies have documented only limited or no therapeutic activity of PD-1–blocking therapies in mCRPC; however, several clinical trials currently are investigating the use of such therapies in prostate cancer further. PD-L1 expression on tumor cells has been shown to be associated with response to anti-PD-1 therapies⁵ and subsequently has been established as a predictive biomarker that is clinically useful in non–small-cell lung cancer.³⁷ Although a small subset of tumors show constitutive up-regulation of PD-L1 expression owing to genomic alterations involving the PD-L1 gene locus,^{38,39} the majority of cancers (including prostate cancer) likely show PD-L1 up-regulation as an adaptive response to changes in the immune microenvironment.^{10,12,40} In general, it has been noted that the reproducibility of biomarker studies evaluating PD-L1 expression has been challenging owing to high interlaboratory and interobserver variation. The use of divergent, often insufficiently validated, antibodies and non-standardized scoring systems has complicated the interpretation and comparison of previously published studies. These analytic differences likely have contributed to discordant results regarding PD-L1 expression in multiple tumor types. Several groups have assessed the expression of PD-L1 in primary prostate cancer specimens using

different monoclonal antibodies, and the rate of PD-L1 positivity varies greatly between different studies. For instance, Gevensleben et al¹³ reported up to 61.7% of cases showing moderate to strong PD-L1 expression in a large TMA-based cohort. This report also suggested a strong association between high PD-L1 expression and early biochemical recurrence. Although the investigators performed Western blot and flow cytometry–based specificity control experiments, immunohistochemistry-based validation experiments were not performed and this antibody eventually was discontinued by the manufacturer. Another study reported an even higher rate of PD-L1 positivity of 92% in primary prostate cancer, with 59% of cases showing high PD-L1 expression in tumor cells.³⁶ Using two broadly validated antibody clones, three recent studies documented tumor-cell–specific PD-L1 expression in 18 of 130 (14%), 2 of 25 (8%), and 3 of 20 (11%) cases, respectively, in treatment-naïve primary prostate cancer.^{9,11,12} It is important to note that although some antibodies were validated using cell line models and immunoblotting, nonspecific off-target reactivity in human tissues may not be excluded. Therefore, careful cross-comparisons of different PD-L1 antibodies in well-defined case cohorts are necessary to establish the reproducibility of immunohistochemical assays used to detect PD-L1 expression.^{14,37} With increasing mechanistic insights into the molecular basis of response to PD-1/PD-L1 axis blockade, additional biomarkers are being explored that allow for a more accurate prediction of

treatment response, at least in a limited subset of patients. One such emerging biomarker for response to immunotherapy is the total number of mutations present in a tumor specimen, that is, the tumor mutational burden.^{33,41} Along these lines, high response rates to PD-1–targeting therapies were observed in cancers deficient in mismatch repair, which are known to contain exceptionally high numbers of somatic mutations.³³ In the selected cohort, two primary prostate cancer cases with remarkably high PD-L1 expression were identified. These cases did not show evidence of microsatellite instability (Supplemental Figures S1 and S2; Supplemental Tables S1 and S2); however, recent evidence suggests that microsatellite instability PCR using the contemporary markers developed for colorectal carcinoma may be suboptimal for the detection of mismatch repair–deficiency prostate cancers.⁴² It therefore remains to be seen which genetic, epigenetic, or microenvironmental factors contribute to the high-level expression of PD-L1 in a very small subset of primary prostate cancers.

Interestingly, a recent study showed that prostatic duct adenocarcinoma, which is morphologically distinct from the more common acinar adenocarcinoma of the prostate, frequently shows mismatch repair gene alterations and associated hypermutation phenotypes.⁴³ PD-L1 expression therefore was evaluated in a cohort of 24 prostatic duct adenocarcinomas and 5 of 24 cases (21%) showed detectable PD-L1 immunolabeling.

Based on prior *in vitro* data, it was suggested that blockade of the androgen axis and conversion to castration resistance may be associated with increased expression of PD-L1.²¹ Results from a neoadjuvant trial in primary prostate cancer, however, suggested a trend toward lower PD-L1 expression in men receiving neoadjuvant hormonal therapy.⁹ To date, however, there are no comprehensive data published on PD-L1 expression in mCRPC. To address this knowledge gap, both biopsy as well as surgical resection specimens of men with mCRPC who were treated with contemporary androgen-deprivation therapy, with the majority of patients receiving enzalutamide or abiraterone acetate, were analyzed (Supplemental Table S2). Importantly, in this cohort of treatment-refractory mCRPC, low-level expression (1% to 5% of tumor cells positive) in 6 of 18 cases (33%) and somewhat higher level expression (>5% of tumor cells positive) in 2 of 18 cases (11%) showing PD-L1 immunolabeling in >5% of tumor cells was found. This finding indicates that PD-L1 expression levels appear to be increased in mCRPC compared with hormone-naïve primary carcinoma. In addition, using a cohort of surgical resection specimens of metastases from men with mCRPC for which diagnostic slides sampling an entire cross-section of the lesion were available, a similar distribution of PD-L1 positivity with low-level expression (1% to 5% of cells positive) in three of nine cases (33%) and >5% of tumor cell positivity in one of nine cases was observed. These data suggest that at least in this cohort of similarly treated metastatic lesions, no differences in PD-L1 expression levels

were observed between core biopsy and surgical resection material.

This study had a few limitations. First, the majority of cases evaluated in this study were represented by two to four tissue cores on TMAs. Given the heterogeneity and often limited focal expression of PD-L1 in tumor cells, it is likely that the frequency estimates of PD-L1 positivity may represent an underestimation. Indeed, a high level of intratumor heterogeneity in PD-L1 expression was seen in cases of resected distant metastases (Supplemental Figure S4). In addition, the majority of TMAs were constructed from archival formalin-fixed, paraffin-embedded tissues, and it is unclear if long-term storage of tissue material may result in a loss of PD-L1 immunoreactivity. However, in this study, TMAs containing recently collected formalin-fixed, paraffin-embedded tissues were included, which showed no difference in the level of PD-L1 immunoreactivity compared with older archival tissues (data not shown). Importantly though, a large number of recent biopsy specimens from distant soft-tissue metastases were included, which are most representative of tissue samples used for clinical decision making. Immunoreactivity was scored in epithelial cancer cells and signals from tumor-associated immune cells were not counted. Neoplastic epithelial cells were identified based on histomorphologic features and co-immunolabeling was not performed to identify individual cell lineages. It is important to note that based on these criteria the vast majority of immunostaining was restricted to cancer cells. However, it cannot be excluded that a small fraction of PD-L1 immunoreactivity was contributed by immune cells in close proximity to cancer cells. Because of technical challenges, biopsy specimens from bony lesions were not included. It therefore remains to be shown if metastases to the bone show a different PD-L1 expression pattern.

In conclusion, this study presents the most comprehensive evaluation of PD-L1 expression in both primary and metastatic prostate cancer. Furthermore, this study documents that PD-L1 expression is present in a small subset of primary prostate cancer, and is increased in mCRPC.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.02.014>.

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