PD-L1 expression in tumor metastasis is different between uveal melanoma and cutaneous melanoma

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Aim: To compare PD-L1 expression between metastatic uveal melanoma (MUM) and metastatic cutaneous melanoma (MCM). **Materials & methods:** A total of 295 MCM and 78 MUM specimens were analyzed for tumor cell PD-L1 expression. Additionally, 91 MCM and 45 MUM specimens were analyzed for PD-1 expression on tumor-infiltrating lymphocytes. **Results:** A total of 77/295 (26.1%) MCM specimens expressed PD-L1 as compared to 4/78 (5.1%) MUM specimens ($p < 0.0001$). PD-1 expression on tumor-infiltrating lymphocytes was greater in MCM (73.6%; 67/91) than in MUM (51.1%; 23/45), respectively ($p = 0.009$). **Conclusion:** Significant differences exist in PD-L1 expression between MCM and MUM. The lower PD-L1 expression in MUM may provide a rationale for failure of PD-1 inhibitor therapy and suggests that immune evasion in this disease may occur via alternative mechanisms.

First draft submitted: 31 May 2017; Accepted for publication: 12 October 2017; Published online: 29 November 2017

Uveal melanoma originates from melanocytes in the choroid, ciliary body and iris of the eye [1]. It is the most common primary intraocular malignancy in adults and occurs almost exclusively in Caucasians, with an incidence of approximately 5–7/million/year in certain European populations [2]. In the USA, it represents about 5% of all melanoma diagnoses [1,3]. Uveal melanoma tends to recur in approximately 50% of the cases despite successful therapy of the primary tumor, in particular those whose tumors have certain risk factors for metastasis, such as large tumor size, ciliary body location, epithelioid cell morphology, high mitotic count and chromosomal abnormalities (e.g., chromosome 3 loss, 8q gain) [4]. Compared with cutaneous melanoma, uveal melanoma is characterized by its distinct genetic profile and clinical presentation, where most metastases are detected in the liver [5–10]. Once uveal melanoma metastasizes, prognosis is poor because metastatic uveal melanoma (MUM) tends to be widespread within the liver and only surgically resectable in some cases [11,12]. Therefore, there is an unmet need for investigating effective systemic therapies.

Systemic immunotherapy with checkpoint inhibitors has vastly improved the treatment of metastatic cutaneous melanoma (MCM), leading to long-term durable responses [13,14]. MUM patients are often treated with immune checkpoint inhibitors identical to those used in the treatment of MCM (anti-programmed death-1 [PD-1] and anti-CTLA4 [Cytotoxic T-lymphocyte-associated protein 4] antibodies), either as monotherapy or in combination. Contrary to MCM, MUM demonstrates a poor response to these immune checkpoint blockades [15] (Table 1) [16–23]

One of the mechanisms by which tumors evade the immune system is through upregulation and expression of PD-L1 protein on their surface. PD-L1 on tumor cells interacts with PD-1 receptor expressed on activated T cells, leading to T-cell exhaustion, anergy and ultimately, immune response abandonment [24,25]. Inhibition of the PD-1/PD-L1 interaction is the mechanism by which anti-PD-1 antibodies induce T-cell activation and antitumor effect.

We evaluated the expression of PD-L1 protein on tumor cells in biopsy samples from MUM patients. We also evaluated PD-1 receptor expression on tumor-infiltrating lymphocytes (TILs). PD-L1 expression on tumor cells and PD-1 expression on TILs from MUM samples were compared with that of MCM specimens. We sought to use

Table 1. Immunotherapy with checkpoint inhibition in metastatic uveal melanoma.			
Study (year)	Type of checkpoint inhibitor therapy used	Results	Ref.
Algazi et al. (2016)	Pembrolizumab (n = 40) Nivolumab (n = 16)	Median PFS: 2.6 months Median OS: 7.6 months	[16]
Kottschade et al. (2016)	Pembrolizumab (n = 10)	Median PFS: 18 weeks	[17]
Tian et al. (2016)	Either nivolumab or pembrolizumab (n = 9)	CR: 0%; PR and SD seen in 2 (25%) and 3 (37.5%) patients, respectively	[18]
Piperno-Neumann et al. (2016)	Either nivolumab or pembrolizumab (n = 21)	OR: 0% Median PFS: 3 months	[19]
Zimmer et al. (2015)	Ipilimumab in pretreated (n = 45) and treatment-naïve (n = 8) patients	Median OS: 6.8 months; median PFS: 2.8 months (95% CI: 2.5–2.9)	[20]
Rodriguez et al. (2014)	Ipilimumab (first line), 13/31 patients evaluable	1/13 PR (7.7%) 6/13 SD (46.2%)	[21]
Maio et al. (2013)	Ipilimumab in pretreated patients (n = 82)	Median OS: 6 months Median PFS: 3.6 months	[22]
Luke et al. (2013)	Ipilimumab (n = 39)	Median OS: 9.6 months	[23]
CR: Complete response; OS: Overall survival; PFS: Progression-free survival; PR: Partial response; SD: Stable disease.			

the comparison with MCM to provide further insight into the distinct immune-biology of MUM and investigate its limited response to treatment with anti-PD-1 antibodies.

Methods

MCM and MUM specimens considered viable, containing sufficient tumor and profiled between April 2016 and March 2017 were included in this retrospective analysis. We analyzed 295 MCM and 78 MUM biopsy specimens for PD-L1 expression. Out of these, 91 MCM and 45 MUM specimens were also evaluated for PD-1 expression on intratumoral TILs. All MUM liver specimens included, displayed a nodular growth pattern. Diagnosis of metastatic melanoma was confirmed in all specimens by a board-certified pathologist at a CLIA-certified pathology laboratory (Caris Life Sciences, AZ, USA).

All data for the analysis were obtained from a commercial database, a clinical laboratory system that stores all laboratory results ordered by physicians at Caris Life Sciences. De-identified data created under the Caris honest-broker policy followed consultation with the Caris Life Sciences IRB of record (Western IRB). Upon review of the protocol, the Caris Human Subject Compliance Officer confirmed the honest-broker policy and verified all data as a de-identified dataset.

PD-L1 immunohistochemistry

Formalin-fixed paraffin-embedded tissues from 295 MCM and 78 MUM patients were investigated for PD-L1 expression (SP142, Ventana Medical Systems, AZ, USA) using automated immunohistochemical methods at a CLIA/CAP/ISO15189-certified lab (Caris Life Sciences). Cases were considered positive for PD-L1 expression when ≥ 5% membranous staining of tumor cells was detected with 2+/3+ intensity [26].

PD-1 immunohistochemistry

Expression of intratumoral PD-1 on TILs was determined using an anti-PD-1 monoclonal antibody (NAT105, Cell Marque, CA, USA), with density assessed as previously described [27]. Tissue slides were reviewed at low power (2–4×) for identification of an area with the highest number of PD-1+ cells. This ‘hot spot’ area was then examined under a high power field (40×) to identify and count the number of PD-1+ TILs [27]. PD-1 positivity was determined by counting one or more PD-1+ TILs in a high power field. All specimens evaluated for PD-1 expression had been tested for PD-L1 expression.

Statistical methods

A chi-square test was used for comparison of variables and groups. Differences were considered significant at a p-value < 0.05. All data were analyzed using IBM SPSS v24 (IBM Corp, NY, USA).

Table 2. PD-L1 expression on metastatic melanoma cells.		
Site of metastasis	Number of patients (n)	PD-L1 expression
<i>Uveal melanoma</i>		
Liver	64	2/64 (3.1%)
Lung	4	0%
Other [†]	10	2/10 (20%)
	Total: 78	4/78 (5.1%)
<i>Cutaneous melanoma</i>		
Liver	27	4/27 (14.8%)
Lung	49	17/49 (34.7%)
CNS	40	12/40 (30.0%)
Lymph node	147	35/147 (23.8%)
Other [‡]	32	9/32 (28.1%)
	Total: 295	77/295 (26.1%)

[†]Soft tissue (n = 3), bone (n = 2), gastric (n = 2), lymph node (n = 1), CNS (n = 1) and thyroid (n = 1).
[‡]GI tract (duodenum, gastric, colon and gallbladder) (n = 19), bone (n = 7), adrenal (n = 4) and spleen (n = 2).

Figure 1. Patterns of PD-L1 expression; cases considered positive when $\geq 5\%$ membranous staining of tumor cells was detected with 2+/3+ intensity. (A) Uveal melanoma metastasis in thyroid tissue (PD-L1⁺) (B) Uveal melanoma metastasis in the liver (PD-L1⁻); (C) Cutaneous melanoma metastasis in the lung (PD-L1⁺); and (D) Cutaneous melanoma metastasis in the liver (PD-L1⁻).

Results

Overall, 77/295 (26.1%) MCM specimens expressed PD-L1. In comparison, only 4 of the 78 MUM specimens (5.1%) demonstrated PD-L1 expression: 26.1 versus 5.1%; $p < 0.0001$; (Table 2, Figure 1 A–D). Specific to MCM, the highest rate of PD-L1 expression was identified in lung (34.7%) and CNS (30.0%) metastatic specimens. Cutaneous melanoma specimens that had metastasized to the liver showed the lowest rate of PD-L1 expression at 14.7% of specimens evaluated. In MUM, two PD-L1⁺ specimens were from the liver while the other two involved the thyroid gland and peri-gastric tissue.

Table 3. PD-1 expression on tumor-infiltrating lymphocytes at different metastatic sites.		
Site of metastasis	Number of patients (n)	PD-1 expression
Uveal melanoma		
Liver	39	19/39 (48.7%)
Other [†]	6	4/6 (66.6%)
	Total: 45	23/45 (51.1%)
Cutaneous melanoma		
Liver	6	3/6 (50.0%)
Lung	20	16/20 (80.0%)
CNS	19	15/19 (78.9%)
Lymph node	36	26/36 (72.2%)
Other [‡]	10	7/10 (70.0%)
	Total: 91	67/91 (73.6%)
[†] Soft tissue (n = 2), bone, gastric, lymph node and lung (n = 4).		
[‡] GI tract (duodenum, gastric and colon) (n = 5), bone (n = 2), spleen (n = 2) and adrenal (n = 1).		

Table 4. Correlation between PD-L1 expression and PD-1 expression.		
	PD-L1 ⁺	PD-L1 ⁻
Metastatic cutaneous melanoma		
PD-1 ⁺ (n = 67)	21	46
PD-1 ⁻ (n = 24)	1	23
Metastatic uveal melanoma		
PD-1 ⁺ (n = 23)	1	22
PD-1 ⁻ (n = 22)	0	22

A higher rate of PD-1⁺ TILs was seen in MCM (n = 91) as compared with MUM (n = 45) (73.6 vs 51.1%; p = 0.009); (Table 3). All specimens evaluated for PD-1 expression had been tested for PD-L1 expression. A total of 44 of the 45 MUM samples that were analyzed for PD-1 expression on TILs tested negative for PD-L1 expression on melanoma cells. Out of these, 50% were PD-1⁻/PD-L1⁻ (n = 22); the other 50% were PD-1⁺/PD-L1⁻ (n = 22), (Table 4). Only one MUM specimen (from peri-gastric tissue) was PD-L1⁺/PD-1⁺. For the 91 MCM specimens that were tested for both PD-L1 and PD-1 expression, 95.4% (21/22) of the specimens that tested positive for PD-L1 were also positive for PD-1 expression (Table 4).

Discussion

PD-1 (CD279) is an inhibitory transmembrane protein that binds to PD-L1 (B7-H1) and PD-L2 (B7-H2). PD-1 is differentially expressed on T cells (CD4⁺, CD8⁺), B cells, NK cells and activated monocytes [28]. PD-L1 is expressed on a variety of tumor cells [29], as well as on nonmalignant hematopoietic cells (APCs, peripheral blood monocytes and dendritic cells) and nonhematopoietic cells [24,30]. The interaction of PD-1 on activated T cells with PD-L1 leads to T-cell apoptosis and suppression of T-cell-receptor-mediated lymphocyte proliferation and cytokine secretion [24,25]. Tumor cells upregulate cell-surface PD-L1 expression as a mechanism to suppress a T-cell-mediated immune response [25].

PD-L1 expression has been well established in MCM using several different assays [27,29]. Regarding PD-L1 expression in MUM, only limited data from *in vitro* cell line analysis are available [31]. Moreover, assessing *in vitro* PD-L1 expression on uveal melanoma cell lines may not be representative of *in situ* PD-L1 expression within the tumor microenvironment.

Our study is the first large study to report *in situ* expression of PD-L1 in MUM and compare it with PD-L1 expression in MCM. The limited expression of PD-L1 on tumor cells in MUM compared with MCM using the same validated anti-PD-L1 antibody provides an interesting comparison that has never been reported. Additionally, subsets of MCM and MUM assessed for PD-1 expression on TILs also demonstrated a lower expression rate in MUM compared with MCM.

The lack of PD-L1 expression in MUM is unlikely due to a loss of function specific to the tumor. Studies show that uveal melanoma cells are capable of upregulating PD-L1 protein that is functionally effective in causing T-cell inactivation [31–33]. PD-L1 expression on tumor cells in melanoma is typically seen in the presence of activated TILs that express PD-1 [34,35]. More specifically, PD-L1 expression is induced by IFN- γ produced by activated CD8⁺ cells [36]. Thus, PD-L1 expression in melanoma is reflective of an active interaction of the tumor with incoming adaptive immune cells rather than due to constitutive PD-L1 expression intrinsic to the tumor.

Low PD-L1 expression in MUM may be attributed to a combination of systemic immunologic ignorance as well as local immune tolerance at the site of metastasis causing suppression of TIL function. Out of the MUM samples that were analyzed for both PD-1 on TILs and PD-L1 on tumor cells, two predominant patterns of PD-1/PD-L1 expression were seen: PD-1⁺/PD-L1⁺ (suggestive of systemic immunologic ignorance, whereby there is a lack of activated TILs in the microenvironment) and PD-1⁺/PD-L1[−] (suggestive of local immune tolerance where activated TILs are functionally suppressed at metastatic sites). Similar combinations were also seen in MCM. However, the PD-L1⁺/PD-1⁺ subgroup was much more dominant in MCM group (21/91; 23.1%) compared with that of MUM (1/45; 2.2%), which indicates a different immune microenvironment between these two melanoma types (Table 4).

We demonstrated less PD-1 expressing TILs in MUM compared with MCM. This may be explained by immunologic ignorance of circulating adaptive immune cells that are unable to detect the relatively less immunogenic uveal melanoma tumor likely due to its low mutational burden [7]. Decreased cytotoxic T cells trafficking into the tumor microenvironment would result in lower IFN- γ production that might explain the lack of PD-L1 expression in MUM.

It is plausible to partially attribute low PD-L1 expression in MUM in the absence of TILs due to a failure of systemic immune surveillance. However, we also demonstrated the PD-1⁺/PD-L1⁺ subgroup in MUM. Uveal melanoma is known to have TILs both in the form of CD4⁺ and CD8⁺ T cells at metastatic sites in the liver. Although a subset of these TILs in MUM is immunologically competent in eliciting antitumor response, this subset seems to occur much less frequently as compared with MCM where significantly large proportions of TILs are CD8⁺ cells that demonstrate potent antitumor activity [37]. The relative abundance of functionally competent TILs in MCM is able to engage tumor cells more effectively, perhaps that is why the PD-1⁺/PD-L1⁺ subgroup was demonstrated in MCM, while it was almost absent in MUM. The rarity of a PD-1⁺/PD-L1⁺ subgroup in MUM can be due to several possible reasons. It is possible that TILs in MUM with effector antitumor function are a minority within a predominant population of regulatory lymphocytes and are thus unable to effectively target melanoma cells and induce PD-L1 expression on tumor cells. Another explanation could be a failure of immunologically competent TILs in MUM to overcome additional immune-tolerant barriers within its microenvironment that are specific to uveal melanoma's origin in the eye and its site of metastasis, the liver.

The exact mechanisms via which uveal melanoma suppresses and evades an adaptive immune response at its site of metastasis are yet to be elucidated. It is conceivable that uveal melanoma might have immune privilege owing to its unique disease biology that is distinct from that of cutaneous melanoma. Uveal melanoma originates in the potent immune-suppressive environment of the eye. The eye itself is a site of immune privilege and utilizes several cellular and cytokine-mediated mechanisms to protect itself from an innate and adaptive immune attack [38]. It is believed that uveal melanoma cells retain and employ these mechanisms at metastatic sites to evade immune surveillance and cytotoxic effect. The liver, the preferred site of metastasis may confer additional protection. It provides an ideal immune-sanctuary site for tumor growth by its ability to locally mediate T-cell inactivation, tolerance and apoptosis [39]. For instance, the liver is rich in tryptophan 2,3-dioxygenase that degrades tryptophan along the kynurenine pathway, which might explain a local T-cell suppressive immune microenvironment [40]. Indeed, clinical studies in cutaneous melanoma have shown relatively poor responses to anti-PD-1 therapy in the setting of liver metastasis, with the liver metastasis also demonstrating lower rates of PD-L1 expression [41,42]. Interestingly, most liver metastasis in our study showed absence of PD-L1 expression, including 62/64 (96.8%) MUM samples and 23/27 (85.2%) MCM samples (Table 2). This suggests that the variation in PD-L1 expression might also be site specific and not just tumor specific. Further analysis of such site-specific variation in PD-L1 expression in metastatic cancer is warranted and should be explored in prospective clinical trials.

The role of PD-L1 expression as a predictive biomarker in cancer immunotherapy remains controversial. However, the abundance of PD-L1 or lack thereof can provide information regarding the diverse immune signature of tumors. We demonstrated a significant difference in PD-L1 expression on tumor cells between MUM and MCM. Our results infer that in most instances uveal melanoma does not utilize the PD-1/PD-L1 axis to avoid an immune

attack, thus offering an explanation for its poor response to treatment with anti PD-1 therapy. However, a lack of PD-L1 expression does not mean that uveal melanoma is ‘immunotherapy resistant’. Indeed, immunotherapeutic interventions like adoptive T-cell transfer seems promising for control of metastatic disease in uveal melanoma [43]. Our study is limited by the number of samples assessed for PD-1 expression on TILs as well as the lack of characterization of TILs to identify specific lymphocyte subsets. Data on characterization of TILs in MUM is lacking and will be crucial to the development of novel therapeutic interventions. Continued investigation of the immune biology of uveal melanoma is warranted to identify alternative mechanisms of immune escape.

Conclusion

Despite having a similar melanocytic cell of origin, significant differences exist in PD-L1 expression on tumor cells between MUM and MCM. Low PD-L1 expression rates on MUM cells might explain the lack-of-treatment response to anti-PD-1 therapy observed in this disease, suggesting that the tumor most likely evades the immune system via alternate immune evasive mechanisms that should be explored and targeted.

Summary points

- PD-L1 expression on melanoma cells is significantly lower in metastatic uveal melanoma (MUM) as compared with metastatic cutaneous melanoma (MCM).
- Low PD-L1 expression in MUM likely explains its lack of response to treatment with anti PD-1 immune check-point inhibitors.
- MUM also seems to demonstrate lesser PD-1 expressing tumor-infiltrating lymphocytes as compared with MCM.
- Tumor cells in melanoma liver metastasis (both MUM and MCM) tend to demonstrate low PD-L1 expression. This observation should be further explored in prospective studies investigating immune check-point inhibitor therapy to assess the utility of such treatments in the setting of liver metastasis.
- Studies of novel immunotherapeutic interventions in MUM should include a focus on developing modulators of the immune-suppressive environment of the liver.

Acknowledgements

Shingo Sato for assisting with graphics designing and formatting.

Financial & competing interests disclosure

D Arguello, C Johnston and Z Gatalica are employees of Caris Life Sciences and have stock ownership in the company. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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