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Nodular primary cutaneous melanoma is associated with PD-L1 expression

Background: In previous studies, patients with Stage III melanomas expressing PD-L1 in more than 5% of their neoplastic cells had improved recurrence-free survival with anti-PD1 adjuvant therapy. *Objectives:* We examined PD-L1 expression as a possible biomarker of primary cutaneous melanomas in the vertical growth phase. *Materials and Methods:* This was a retrospective study including 66 patients with invasive primary cutaneous melanomas. We assessed patient clinical and histopathological data and performed immunohistochemical assays with melanoma specimens from the patients to evaluate PD-L1, PD-1, CD3, CD8 and FoxP3 expression. *Results:* We observed PD-L1 expression in 21% (14/66) of our samples, and this expression correlated with increased melanoma thickness ($p = 0.002$) and nodular-type melanoma ($p = 0.001$). After adjusting for tumor thickness using a logistic regression test, the association of PD-L1 with nodular-type melanoma persisted. Nodular-type melanoma was 6.48 times more likely to be positive for PD-L1 than other histological types ($p = 0.014$; 95% CI: 1.46-28.82). As expected, PD-L1 expression correlated with the number of PD-1-expressing cells in the tumor-infiltrating lymphocyte population ($p = 0.04$). No correlation with PD-L1 was observed for age, sex, tumor site, skin phototype, ulceration status, sentinel lymph node status, metastasis development or survival. Regarding the immune profile of the tumor-infiltrating lymphocytes of PD-L1-positive and -negative groups, no significant differences were observed in the numbers of CD3+, CD8+FoxP3-, CD8-FoxP3+ and CD8+FoxP3+ cells by immunohistochemistry. *Conclusion:* Nodular-type melanoma is associated with PD-L1 expression and may be a suitable candidate for adjuvant therapy of primary melanomas treated with immunotherapy.

Key words: nodular melanoma, PD-L1, PD-1, CD3, CD8, FoxP3, tumor infiltrating lymphocytes, Breslow index, tumor microenvironment

The achievement of prolonged recurrence-free survival (RFS) using immunotherapy as an adjuvant therapy, encourages researchers to explore when patients with melanoma should start adjuvant therapy and who should receive it, as immunotherapy is an expensive treatment that can have great toxicity [1, 2].
According to databases from countries with a high incidence of melanomas, tumors with a thickness < 2 mm are the most frequent primary melanomas, and they are predicted to account for the majority of deaths in the future [3, 4]. Thus, there is a rationale for the use of adjuvant immunotherapy in primary melanomas. However, how to determine who would benefit from this approach remains unclear.
The program cell-death 1 (PD-1) protein is expressed in activated T and B lymphocytes, and NK cells inhibiting the immune response. Program cell-death ligand 1 (PD-L1), a cell-surface molecule present in some tumors and antigen-presenting cells, binds to the receptor PD-1 on T lymphocytes and plays a critical role in suppressing the immune response [5]. Recently, one study showed that of patients with melanomas undergoing immunotherapy, those with ≥5% PD-L1 expression had greater RFS than those with <5% PD-L1 expression [1].
In this context, to identify more suitable candidates for adjuvant therapy among primary melanomas, we aimed to analyze the differences in PD-L1 expression in vertical growth-phase primary melanomas according to demographic, clinical and histopathological aspects as well as the immune profile of tumor-infiltrating lymphocytes (TILs).

Materials and methods

This was a retrospective study of a cohort of 66 patients with primary cutaneous melanomas in the vertical growth phase. Data were collected from the Division of Dermatology, University of São Paulo, Brazil. This work has been approved by the University of São Paulo, Brazil

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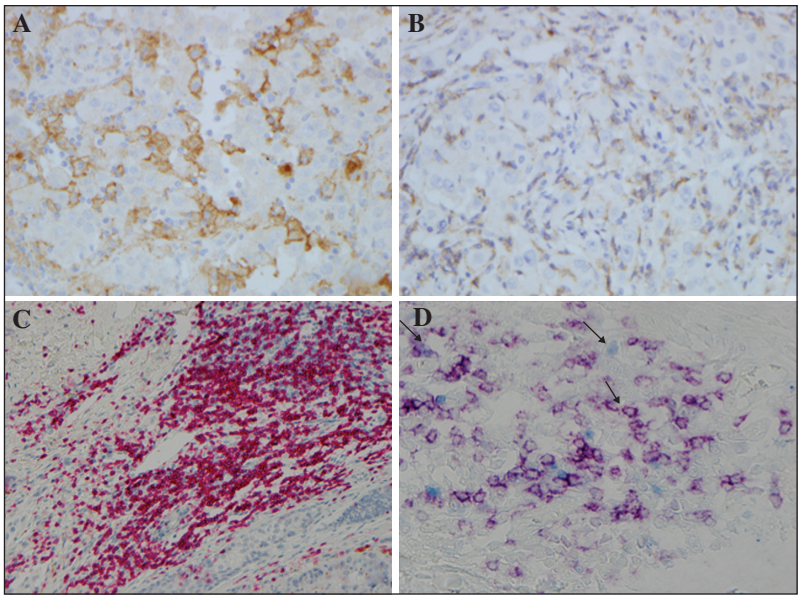

Figure 1. Immunohistochemistry panel for detection of PD-L1, PD-1, and CD3+ cells and CD8 FoxP3 double staining. **A**) PD-L1 in melanoma cells; only membranous stain was considered positive, although cytoplasm stain can also be seen. **B**) PD-1 stain is a membranous marker, mainly in TIL. **C**) CD3+ cells (red) in TIL of melanoma. **D**) CD8 + FoxP3- cells show a purple membranous stain, while CD8-FoxP3+ cells show only light blue nuclei, and CD8 + FoxP3+ cells reveal light blue nuclei with a purple membrane (arrows).

(7659137.0.3001.0065) and Partners Institutional Review Board (2013P000172) of Harvard University, Boston, USA. Our data included sex, age, phototype, tumor location, sentinel lymph node status, metastasis development, melanoma-specific survival, thickness, tumor subtype and ulceration status, and TIL grade, as described previously [6].

Immunohistochemical (IHC) detection of all markers was performed with formalin-fixed, paraffin-embedded tissue sections (*figure 1*). For PD-L1, we used a mouse monoclonal antibody clone, 405.9A11 (Dana Farber Hospital, USA). Melanomas that had membrane staining of more than 5% of the tumor cells were considered positive (*figure 1A*). PD-1 and CD3 immunohistochemical detection was performed in the same manner, using the antibody, Nat105, from Cell Marque and the Leica Biosystem DAB kit (Cat #DS9800) and LN10 from the Leica Bond Polymer Refined Detection Kit (Cat# DS9800) and Bond Polymer Red Detection Kit (Cat# DS9390), respectively. CD8 and FoxP3 double staining utilized the antibodies SP16 (Biocare Medical) detected with Red Permanent chromogen (DAKO) and FJK-16 s (eBioscience) using Blue substrate (Vector Labs), respectively (*figure 1B-D*). Tonsil tissue was used as a positive control [7]. We adapted the morphometric point-counting method to evaluate the volume density of lymphocyte subsets [8, 9]. For PD-1-positive cells, we used the same *modus operandi*, but we counted all cells present within a 10 × 10-mm area.

Associations between PD-L1 expression and continuous variables were evaluated using a two-tailed Student's t test. Associations between PD-L1 expression and categorical variables were analyzed by Fisher's exact test. One-way ANOVA was used to test Breslow groups, melanoma subtypes, tumor sites, patient follow-ups and TIL grades. Logistic regression was used to test melanoma subtypes, and hazard ratios (HRs) were calculated, adjusting for Breslow index. The Kaplan-Meyer method was used for survival analyses. Statistical analysis was performed with using Graph Prism 6.0 software, and p value < 0.05 was considered statistically significant.

Results

The majority of our patients were women (40/66) with melanoma on the trunk and extremities (36% each). The age ranged from 23 to 86 years old (mean: 56.3), and the tumor thickness from 0.3 to 14.4 mm. The PD-L1 protein was expressed by melanoma cells in 21% (14/66), with difference among Breslow groups ($p = 0.013$) (*table 1*). The mean tumor thickness of the PD-L1-positive group was greater than that of the PD-L1-negative group (6.3 mm +/- 0.93 vs. 3.1 mm +/- 0.43, respectively; $p = 0.002$) (*figure 2*). PD-L1 expression was not correlated with sex, age or tumor site.

Nodular melanoma was the most frequent histological type (38%), followed by superficial spreading melanoma (35%), acral lentiginous and lentigo maligna melanoma (9% each). PD-L1 was expressed significantly more frequently in nodular melanoma ($p = 0.001$) (*table 1*). Hence, we used logistic regression to analyze the presence of PD-L1 in nodular melanoma, adjusting for tumor thickness. This analysis showed that the chance of PD-L1 positivity was 6.48 times greater in nodular-type melanoma than in other types (95% CI: 1.46 - 28,82; $p = 0.014$).

Ulceration was observed in 20% of patients. Sentinel lymph node biopsy was performed in 18% of patients with 25% positivity, showing no association with PD-L1.

The mean follow-up period was 108 months (7-264 months). A Kaplan-Meyer disease-specific survival curve showed no difference between PD-L1-positive

Table 1. Clinical and histopathologic parameters and PD-L1 expression in primary cutaneous melanomas.

Parameter	Subgroup	Number of patients (%)	PD-L1 positive *n* (%)	PD-L1 negative *n* (%)
Gender	Male	26 (39)	4 (29)	22 (71)
	Female	40 (61)	10 (25)	30 (75)
Mean Age (years)		56.3	52.4	57.4
Location	Extremity	24 (36)	4 (17)	20 (83)
	Trunk	24 (36)	8 (33)	16 (67)
	Head and neck	12 (18)	2 (17)	10 (83)
	Acral	6 (10)	0 (0)	6 (100)
Melanoma subtype	Superficial spreading	23 (39)	1 (4)	22 (96)
	Nodular	25 (33)	11 (44)*	14 (66)*
	Lentigo maligna	6 (9)	1 (17)	5 (83)
	Acral lentiginous	6 (10)	0 (0)	6 (100)
	Unclassified	6 (9)	1 (17)	5 (83)
Ulceration	Present	15 (20)	6 (40)	9 (60)
	Absent	51 (80)	8 (16)	43 (84)
Breslow#	≤ 1.0 mm	13 (20)	0 (0)	13 (100)
	1.01-2.0 mm	20 (30)	2 (10)	18 (90)
	2.01-4.0 mm	5 (8)	1 (20)	4 (80)
	> 4.0 mm	28 (42)	11(26)	17 (74)
Tumor infiltrating lymphocytes	Absent	10 (15)	1(10)	9 (90)
	Non-brisk	48 (73)	11 (23)	37 (77)
	Brisk	8 (12)	2 (25)	6 (75)
Sentinel lymph node	Positive	3 (11)	1 (33)	2 (67)
	Negative	9 (89)	2 (22)	7 (78)
Follow-up	Disease-free	36 (55)	5 (14)	31 (86)
	Alive with metastases	14 (21)	4 (29)	10 (71)
	Dead of melanoma	13 (20)	4 (31)	9 (69)
	Lost	3 (4)	1 (33)	2 (67)

$p = 0.001$ for nodular type compared to other primary tumor types; # $p = 0.013$ for categorical Breslow groups

and -negative tumors ($p = 0.356$). No anti-PD-1 therapy was performed at that time.

TILs were graded as absent, non-brisk and brisk in 15%, 73% and 12%, respectively. PD-L1 was expressed in 23% of the tumors with non-brisk infiltrates, 25% with brisk, and 10% of tumors without TILs ($p = 0.31$) (*table 1*). We assessed the positive TIL profiles of 57 vertical growth-phase melanomas (*figure 3*). No correlations were found between PD-L1 expression and the numbers of CD3+ T cells, CD8 +FoxP3- T cells, CD8-FoxP3+ T cells, or CD8 + FoxP3+ T cells. PD-L1 expression in melanoma cells correlated with PD-1 expression in TILs ($p = 0.044$) (*figure 4*).

Discussion

PD-L1 expression was present in 21% of primary invasive cutaneous melanomas. We defined 5% expression as the cut-off value because this is the most frequently utilized in association studies. A previous study observed 14% positivity for PD-L1 in primary melanomas [5]. In another study, 21% of desmoplastic melanomas showed ≥ 25% tumor cell staining for PD-L1 [10], and 23% of vulvar melanomas showed ≥ 5% tumor cells positive for PD-L1 [11].

There was an association between PD-L1 positivity and tumor thickness based on both categorical ($p = 0.013$) and

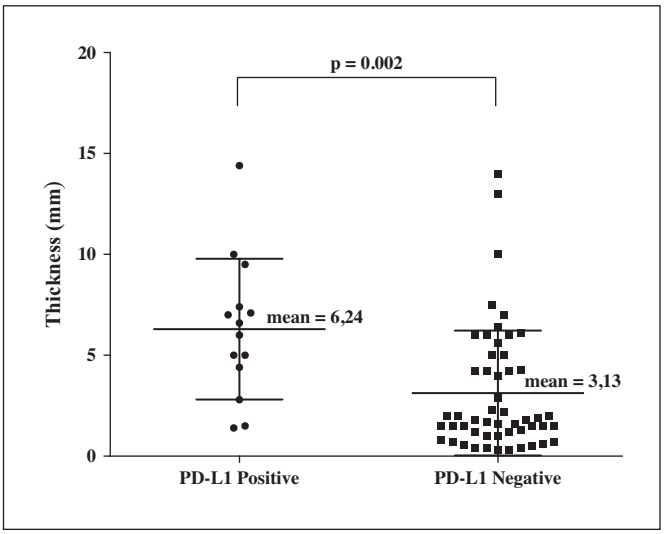

Figure 2. Association between primary cutaneous melanoma thickness in vertical growth phase and PD-L1. Each point represents one patient, and the horizontal bars represent the mean melanoma thickness of each group ($p = 0.002$).

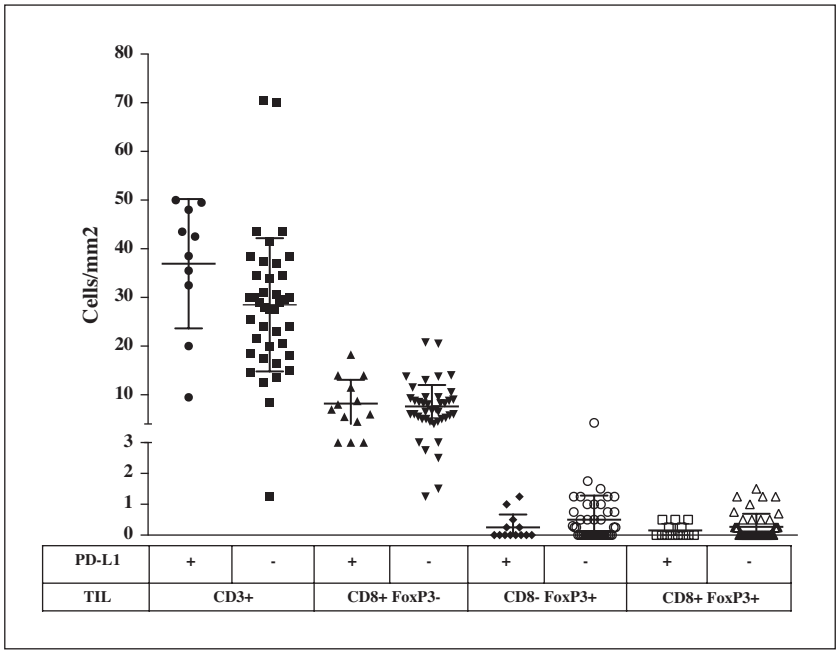

Figure 3. Number of CD3 + , CD8+ FoxP3-, CD8- FoxP3+ and CD8+ FoxP3+ lymphocytes in PD-L1-positive and -negative melanomas. Each geometric figure represents one patient, and the horizontal bars represent the mean value.

continuous (mean thickness of PD-L1-positive tumors was twice that of negative tumors; $p = 0.002$) variables analyses. Kraft *et al* also found a correlation between PD-L1 and thickness in desmoplastic melanomas [10].

We found that PD-L1 correlated with nodular-type melanoma ($p = 0.001$). As far as we know, this is a new finding. As nodular type is one of the most strongly associated with thick melanoma [12] and PD-L1 was found to be associated with thickness, we thought that thickness could be a confounding factor. Hence, we evaluated the results with a logistic regression test adjusted for thickness, which resulted in a 6.48-fold greater chance of having a PD-L1-positive tumor in nodular-type melanoma than in other types. Some authors have stated that nodular melanoma is clinically distinct and the predominant contributor to melanoma-related death [13]. Although the incidence and survival rates of superficial spreading melanoma have increased between 1978 and 2007, neither the incidence nor the survival rate of nodular melanoma has changed [14]. In clinical trials for anti-PD1 drugs, neither PD-1/PD-L1 expression specifically in nodular melanoma nor data on adjuvant treatment regarding RFS for nodular melanoma were investigated.

Could PD-L1 be an innate and/or adaptive mechanism of tumor resistance against the immune response [15]? By analyzing tumor-infiltrating lymphocytes, we observed that

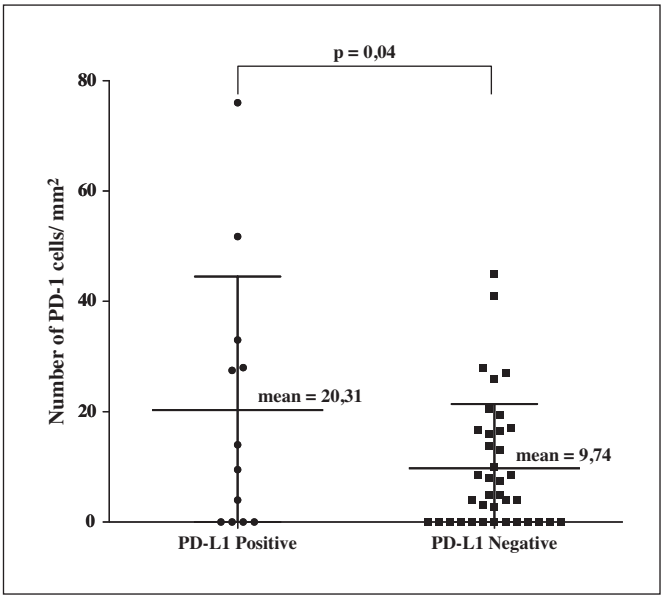

Figure 4. Number of PD-1 cells/mm^2 in PD-L1-positive and PD-L1-negative melanomas. Each point represents one patient, and the horizontal bars represent the mean value ($p = 0.04$).

only 10% of absent-TIL tumors were positive for PD-L1, whereas non-brisk and brisk TIL infiltrates were present in 23% and 25% of PD-L1 positive melanomas, respectively. Although melanomas without TILs showed less PD-L1 expression than TIL-infiltrated melanomas, suggesting that the protein could be expressed as an adaptive resistance during a more vigorous immune response, the difference was not significant ($p = 0.31$). Another study also reported a heterogeneous association between PD-L1-positive melanomas and TIL infiltration [5].If PD-L1 expression has any impact on melanoma prognosis, it could be by modifying the tumor immune environment. The presence of TILs is associated with higher survival rates in melanoma [16-19]. The expression of PD-L1 was an independent risk factor for melanoma-specific survival in one study, with PD-L1 expression being associated with a worse prognosis [20]. In contrast, Taube *et al.* showed improved overall survival in PD-L1-positive metastatic melanomas, but not in primary melanomas [21]. Chlopik *et al*. found a significant correlation between tumor PD-L1 and overall survival based on a univariate analysis, but not multivariate analyses [11]. Thierauf *et al.* described a positive association between PD-L1 expression and recurrence-free survival in mucosal melanomas [22], but PD-L1 expression $\geq$ 25% in desmoplastic melanomas correlated with relatively short disease-free and disease-specific survival [10]. Kakavand *et al*. observed no correlation between PDL1 expression in sentinel lymph node metastases and disease outcomes [23]. Our results did not show an association with overall survival, corroborating the heterogeneity of PD-L1 expression and its association with prognosis.The composition of the lymphocytic infiltrate may have an important role. We assessed specific T-cell subsets in the tumor infiltrate. CD3+ cells have shown to be associated with improved survival in cancer, as reported for TILs in general [24]. Our study showed no difference in the number of CD3+ T cells between PD-L1-positive and PD-L1-negative melanomas. There were no associations between CD8 + FoxP3-, CD8-FoxP3 + , or CD8 + FoxP3+ TILs and PD-L1 expression, which is contrary to the findings for CD8+ and FoxP3+ TILs in desmoplastic and vulvar melanomas [10, 11]. These findings support the theory that PD-L1 expression in melanomas may be part of an adaptive immune response. PD-1 expression on TILs and PD-L1 expression on melanoma cells were associated in the present study, reinforcing that the PD-1/PD-L1 pathway is activated and involved in the immune antitumor response.Providing some insight into frequency, clinical and histopathological parameters, lymphocytic tumor infiltrate composition and tumor cell-immune response interaction drivers may help to select specific subsets of melanoma patients for future immunotherapeutic approaches. Anti-PD-1 therapies achieved a complete response in up to 70% of patients with desmoplastic melanoma [25], a subtype that showed high expression of PD-L1 [10]. Another study with all types of melanomas found a relatively high proportion of complete responders to anti-PD-1 therapy in a subgroup of patients with > 5% PD-L1 expression. According to this rationale and the results observed in our study, we hypothesize that nodular melanoma could be a suitable candidate for adjuvant immunotherapy, as this type shows an association with PD-L1 and a high mortality rate. ■

Disclosures

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