PD-L1 expression in melanocytic lesions does not correlate with the BRAF V600E mutation

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Abstract

PD-L1 expression in melanoma correlates with response to PD-1 pathway-blocking antibodies. Aberrant tumor-cell PD-L1 expression may be oncogene-driven and/or induced by interferon-gamma (IFNγ). Melanomas express PD-L1 in association with tumor-infiltrating lymphocytes (TIL), but the potential contribution of the BRAF V600E mutation (BRAFmut) to induced PD-L1 expression has not been determined. Fifty-two archival melanocytic lesions were assessed for PD-L1 expression, TIL infiltration and BRAFmut simultaneously. IFNγ-induced PD-L1 expression in cultured melanomas was assessed in parallel according to BRAF status. Melanocyte PD-L1 expression was observed in 40% of specimens, and BRAFmut was observed in 42% of specimens, but there was no significant concordance between these variables. Almost all melanocytes displaying PD-L1 expression were observed to be adjacent to TILs, irrespective of BRAF status. TIL(−) lesions were not more likely to be associated with BRAFmut, when compared to TIL(+) lesions. Baseline expression of PD-L1 by melanoma cell lines was virtually nil regardless of BRAFmut status and the intensity of IFN-induced PD-L1 expression in melanoma cell lines likewise did not correlate with BRAF mutational status. PD-L1 expression in melanocytic lesions does not correlate with the BRAFmut. Thus, distinct populations of melanoma patients will likely benefit from BRAF inhibitors vs. PD-1 pathway blockade.

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Keywords
PD-L1; BRAF; melanoma; TIL; adaptive immune resistance

Introduction
Programmed death ligand-1 (PD-L1, B7-H1) expression by antigen presenting cells and parenchymal cells is a normal physiologic mechanism for inducing tolerance and tissue protection via its co-inhibitory receptor programmed death-1 (PD-1) on T cells. Melanoma and other tumors have been shown to co-opt this mechanism, facilitating escape from host immunosurveillance (1). We previously demonstrated that melanocyte PD-L1 expression is observed in proximity to tumor-infiltrating lymphocytes (TIL) secreting interferon-gamma (IFNγ) in melanocytic lesions of various histology and stages (2). These findings suggest that molecules or factors expressed or secreted by immune cells in the tumor microenvironment contribute to PD-L1 expression. Since PD-1 ligation on T cells leads to immune paralysis by inhibiting T-cell receptor (TCR)-mediated lymphocyte proliferation and cytokine secretion (3), and paradoxically TILs may trigger their own down-regulation. This phenomenon in which PD-L1 expression on tumor cells is induced by pro-inflammatory cytokines secreted by TILs, in turn causing T-cell dysfunction via PD-1 ligation, has been termed “adaptive immune resistance” (2). PD-L1 expression was thought to be driven primarily by activation of an oncogene(s) (4), leading to diffuse and constitutive (as opposed to cytokine-induced, regional) expression. Separate models of innate and adaptive tumor resistance based on PD-L1 expression mechanisms have been proposed. In fact, both mechanisms may operate concordantly, and their relative contributions may vary by tumor type.

The molecular mechanisms potentially contributing to oncogene-driven PD-L1 expression varied across tumor types. They include: activation of the PI3K-AKT pathway through PTEN loss or mutations in the PI3K catalytic subunit, as in glioblastoma multiforme, breast and prostate cancer (4, 5); mutations in growth factor receptor pathways such as EGFR in lung cancer (6); and chromosomal amplifications and translocations as in lymphomas (7, 8). Melanomas with BRAF mutation have higher levels of pAKT and lower levels of PTEN (9). In in vitro studies melanoma cells lines that are resistant to BRAF inhibitors demonstrate significantly higher levels of PD-L1 expression via MAPK pathway reactivation, when compared to their non-resistant counterparts (10). These findings raise the possibility that an activated MAPK pathway may play a role in driving innate melanocyte expression of PD-L1. However other in vitro studies have shown that BRAF mutations drive secretion of immunosuppressive cytokines such as IL10, IL6, and VEGF, via STAT-3 signaling in melanoma cells (11), suggesting that TILs in BRAF mutant melanomas could be diminished in numbers, and/or dysfunctional and not capable of mediating adaptive (IFNγ-induced) tumor-cell PD-L1 expression.

In this study, we assessed the relationship between BRAF mutational status and PD-L1 expression in melanocytic lesions. Understanding the relationship between these factors has both mechanistic and practical implications for the design of combinatorial therapies in...
patients with advanced disease. We found no correlation between these two variables, demonstrating that mutation-driven constitutive BRAF activation does not independently drive PD-L1 expression in melanomas, and that distinct biomarkers should likely be applied for identification of patients most likely to respond to BRAFi vs PD-1 pathway blockade.

Methods

Case selection and histologic review

Following Johns Hopkins Institutional Review Board approval, 52 melanocytic lesions of various histologic subtypes and stages from 50 different patients were identified in the Johns Hopkins Hospital surgical pathology archives. There were two nevi from one patient, and a primary melanoma and metastasis from another patient. Melanocytic lesions included 14 nevi (7 common congenital or acquired nevi; 6 Spitz nevi; 1 blue nevus), 23 primary melanomas (12 nodular histologic subtype, 4 desmoplastic or spindled, 5 acral lentiginous, and 2 superficial spreading), and 15 metastases. Slides from each case were reviewed by a pathologist (JMT or NR) to confirm the diagnosis and identify one representative formalin-fixed paraffin-embedded (FFPE) tissue block for additional immunohistochemistry (IHC) and molecular studies. In addition, the presence of TILs and associated histiocytes was scored on a semi-quantitative scale as none (0), mild (rare intratumoral cells, mostly perivascular), moderate (immune cells focally present at periphery of tumor and/or extending beyond immediate perivascular regions in the central region of tumor), or severe (diffuse infiltration or completely surrounding the advancing tumor front), as previously described (2). Scoring was performed blinded to the BRAF mutation status of the tumors examined.

Immunohistochemistry for PD-L1 expression

IHC for PD-L1 expression was performed as previously described (2). In brief, staining was performed with a murine anti-human PD-L1 monoclonal antibody (clone 5H1) at 2 ug/mL according to a standard manual protocol. Percentages of tumor cells exhibiting a membranous (cell surface) PD-L1 expression pattern were scored at 0%, 5%, 10%, and then 10% increments up to 100%, independently by two pathologists (JMT and RAA). Differences in scoring were adjudicated. Cases demonstrating at least 5% expression were considered “positive” for PD-L1.

BRAF mutational analysis

Ten consecutive 10-μm unstained sections were cut from each FFPE tissue specimen. The first and last sections were stained with H&E and examined histologically to ensure persistence of the lesion. Cases with lesions that did not persist were excluded from further evaluation. Tissues were microdissected manually to obtain >50% lesional cells. DNA was extracted using Pinpoint reagents according to the manufacturer’s protocol (ZymoResearch, Orange, CA). Mutation testing for BRAF exon 15 (inclusive of codons 595-601), was performed by pyrosequencing assays at the CLIA-certified JHH Molecular Diagnostics laboratory.
**Cell culture and flow cytometry**

Long-term cultured human melanoma cell lines were established in our laboratories from metastatic lesions and maintained in RPMI 1640 medium with 10% FCS. Before IFN-γ treatment, $5 \times 10^5$ cells were seeded into each well in 6-well plates to reach 50% confluence the next day. Then, IFN-γ (Biogen, Cambridge, MA) was added to a final concentration of 500 IU/ml for 48 hr. Adherent cells were harvested with trypsin and stained with anti-human PD-L1-PE (clone MIH, eBioscience, San Diego, CA) or an isotype control. HLA-ABC (clone W6/32, eBioscience) staining was performed simultaneously as a positive control for the effects of IFN-γ. Samples were acquired on the BD FACS Calibur and the data were analyzed with FlowJo Software (TreeStar, Ashland, OR).

**Statistical analysis**

Concordance between BRAF mutational status and PD-L1 positivity was determined by estimating the percent agreement of BRAF mutant with positive PD-L1 and BRAF wild-type with negative PD-L1, and Cohen’s kappa statistic. The agreement must be at least 90% in order to conclude the dependence of the two measures, and a 95% confidence interval below 90% would suggest lack of dependence. In addition, the frequency of BRAF mutant cases were compared between PD-L1-positive and PD-L1-negative samples using Fisher’s exact test. Correlations between mutational status, TIL scores, and melanocyte PD-L1 expression were assessed using Fisher’s Exact test. The effects of IFN-γ on cultured melanoma lines were assessed with the Wilcoxon matched-pairs test, and the Mann-Whitney test. All tests were 2-sided. P-values <0.05 were considered statistically significant.

**Results**

**BRAF V600E does not correlate with tumor PD-L1 expression or with the presence of TILs in tissue biopsies**

Fifty-two melanocytic lesions were evaluated for the presence of PD-L1 expression, BRAF V600E and TILs. Melanocyte PD-L1 expression was observed in 40% (21/52) of specimens. Specifically, 7% (1/14) of nevi, 43% (10/23) of primary melanomas, and 67% (10/15) of melanoma metastases demonstrated membranous PD-L1 expression by ≥5% of melanocyte tumor cells. TILs (mild, moderate, or severe in intensity) were observed in 58% (31/52) of specimens. In 95% (20/21) of cases with PD-L1 expression, PD-L1 staining was geographically adjacent to TILs (Figure 1). The one exception was a primary melanoma with spindle-cell features, which demonstrated broad membranous PD-L1 expression that was not associated with an immune infiltrate.

BRAF mutational analysis revealed V600E mutations in 42% (22/52) of all specimens. This frequency is on par with those reported in the literature for the diverse histologic subtypes and stages included in this study (12-14). Of the V600E mutations identified, almost all of them were based on the classic T1799A nucleotide substitution. However, the one patient with both a primary melanoma and a metastasis, both lesions demonstrated c. 1799_1800delinsAA, also resulting in V600E at the protein level. Information on PD-L1 expression, the presence of TILs, and BRAF mutational status are provided for each specimen-case in Supplementary Table 1.
We then assessed whether BRAF mutational status correlated with PD-L1 expression on tumor cells, independent of T-cell infiltrates, Figure 2A. While melanocyte PD-L1 expression and the BRAF V600E mutation were each observed in approximately 40% of specimens, there was poor concordance between these two features (56% agreement (95% confidence interval: 41%-70%, kappa statistic = 0.09)), indicating a lack of dependence of the two markers. The fraction of BRAFmut cases among PD-L1(+) samples was not significantly different compared to BRAFmut among PD-L1(-) samples (47.6% (10/21) and 38.7% (12/31), respectively, Fisher’s exact p-value=0.58). Consistent with this finding, a significant association between PD-L1 expression and TILs was independent of BRAF mutational status (p=0.002 in BRAFmut subset; p=0.007 in BRAF wild-type subset; Table 1). When only lesions containing TILs were analyzed, PD-L1(+) TIL(+) specimens were not more likely to be associated with BRAF V600E than PD-L1(+) TIL(+) cases (p=0.71). The BRAF V600E mutation alone also did not correlate with the absence or presence of an immune infiltrate (Fisher’s exact p-value=0.56). In fact, the single tumor specimen that demonstrated broad membranous PD-L1 expression not associated with infiltrating lymphocytes, did not contain BRAF V600E.

**BRAF V600E does not influence the intensity of IFNγ-induced PD-L1 expression on melanoma cell lines**

Our IHC scoring system for PD-L1 expression in human FFPE tumor biopsies records the percentage of positive cells in each specimen but not the expression intensity. To explore the possibility that BRAF V600E might influence the intensity of PD-L1 expression induced by IFNγ secreted from lymphocytes in the tumor milieu, we treated 18 established human melanoma cell lines with IFNγ in vitro. Measuring the intensity of induced cell surface PD-L1 via flow cytometry, we found significant up-regulation of PD-L1 protein expression on both BRAF wild-type and mutant cell lines exposed to IFNγ. There was no significant difference between induced expression levels in 10 BRAF mutant cell lines, versus 8 wild-type cell lines (Figure 2B, Suppl. Table 2). Induction of MHC I expression provided a positive control for IFNγ effects in all cultured melanomas (Suppl. Table2).

**Discussion**

Targeted therapies have emerged over the last few years as promising treatments for patients with metastatic melanoma. Two main groups of drugs have shown exciting results. The first main group of drugs targeted the oncogenic pathways; they include the selective BRAF inhibitors for patients whose melanomas have V600E or V600K mutations. Following treatment with these therapies, the majority of patients demonstrate prompt tumor regression, however drug resistance also develops rapidly (15). The second main group of targeted therapies includes immune-modulating antibodies that block the interaction between inhibitory ligands on tumor cells and their cognate receptors on tumor-specific, activated T cells, such as the PD-L1/PD-1 pathway. In contrast to treatments targeting mutant oncogenes, many patients with advanced metastatic melanoma receiving immune checkpoint inhibitors have demonstrated durable tumor regressions (16-19). Combinations of these two potentially complementary treatment approaches are moving into clinical testing. PD-L1 membranous expression by tumor cells in pre-treatment biopsies has been
shown to enrich for response to either anti-PD-1 or anti-PD-L1 therapies (16, 20), suggesting that a better understanding of factors driving PD-L1 display may help guide patient selection and lead to rational treatment combinations.

Given the distinct mechanisms of action of these two classes of anti-melanoma drugs, one might not expect a correlation between the two biomarkers that predict responsiveness. On the other hand, enhanced Akt signaling, which has been linked to constitutive BRAF activation in melanoma, has been reported in other tumor types to induce PD-L1 expression, suggesting that BRAFmut and PD-L1 expression could correlate with each other. We showed here that there is no correlation between these two variables in melanoma and melanocytic lesions.

PD-L1 expression in melanoma tends to be focal and geographically associated with TILs. It has also been associated with the presence of IFN-γ (2), leading us to postulate an adaptive immune resistance mechanism whereby tumor cells under attack from IFN-γ-secreting T cells develop cell surface PD-L1 expression, thereby evading elimination (2, 20). Additional types of infiltrating immune cells, cytokines, and signaling pathways in the tumor microenvironment are also likely to contribute to PD-L1 expression.

As mentioned above, dysregulated signaling pathways may also play a role in driving PD-L1 expression in cancer. Activation of the PI3K-AKT pathway by PTEN loss has been associated with PD-L1 expression in human glioblastomas, breast and prostate cancers (4, 5) and may influence PD-L1 display in melanoma (21). The most commonly activated oncogenic pathways in melanoma include MAPK (through mutations in NRAS and BRAF) and PI3K/AKT (through PTEN loss). These signaling pathways are not always discrete, and in fact often intersect in multiple ways to influence gene expression driving proliferation, differentiation, and cancer cell survival. As MAPK pathway activation and PTEN loss can be associated in melanoma, we queried whether BRAF V600E might correlate with constitutive PD-L1 expression. Conversely, because adaptive immune resistance appears to be a dominant mechanism driving PD-L1 expression in melanoma, and mutant BRAF can have immunosuppressive effects, we also considered the possibility that tumors containing BRAF V600E might be TIL-poor and hence poor PD-L1 expressers. We did not detect an association between mutant BRAF and PD-L1 expression that was either TIL-dependent or TIL-independent, in biopsies of nevi and melanomas, though we did reaffirm the association between TILs and PD-L1 display. These findings are consistent with recent reports studying cell lines and FFPE specimens from patients with melanoma and lung adenocarcinoma, respectively, that failed to demonstrate an association between BRAF mutation and PD-L1 expression (22, 23). Furthermore, we examined the effects of IFN-γ on the intensity of PD-L1 protein expression on the surface of cultured melanomas and did not observe significant differences in BRAF wild-type cell lines versus mutant cell lines. Additional study of other “non-immune”, melanoma-specific tumor characteristics may be warranted for their potential contributions to PD-L1 expression.

Combinatorial treatment regimens of immune checkpoint blockade with selective BRAF inhibitors in patients with BRAF V600E melanoma remain an exciting prospect for the treatment of advanced disease. Results from studies of in vitro models and post-treatment
tumor samples suggest that selective BRAF inhibitors cause paradoxical MAPK pathway activation in T cells, generating new brisk CD8⁺ T-cell infiltrates and concomitant enhancement of tumor PD-L1 expression (24, 25). Results of the current study suggest that the BRAF V600E mutation does not play a major role in directly modulating PD-L1 expression in melanoma cells. Instead, our results support the notion that immune factors in the tumor microenvironment are the major driver of PD-L1 expression in melanoma, irrespective of the BRAF mutational status of the tumor. Our findings also indicate that application of biomarker assessment for these two treatment modalities should be performed independently when determining prioritization and design of therapeutic regimens for melanoma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Figure 1.
PD-L1 expression by melanocytes is observed in geographic association with TILs but does not depend on BRAF V600E. (A) BRAF V600E melanoma metastatic to the lung with a “moderate” grade of immune infiltrates (yellow arrows) on H&E (hematoxylin and eosin) stain, immediately adjacent to melanocytes demonstrating membranous PD-L1 expression by immunohistochemistry. (B) BRAF V600E melanoma metastatic to lymph node with no significant immune infiltrate and no PD-L1 expression by IHC. Melanin pigment is present in the cytoplasm. Original magnification, 200x, all panels.
Figure 2.
BRAF mutational status in melanoma does not correlate with PD-L1 expression in situ or in vitro. (A). PD-L1 expression and the BRAF V600E mutation were each observed in approximately 40% of cases, but there was no significant concordance between these two parameters (B). Cultured human melanoma cell lines were incubated in the presence or absence of 500 IU/ml of IFN-γ for 48 hr. PD-L1 expression was determined by flow cytometry (anti-PD-L1-PE, clone MIH, eBioscience), and ΔMFI of PD-L1 (mean ± SEM) was calculated. ΔMFI, Mean Fluorescence Intensity of staining with isotype control Ab was subtracted from MFI of staining with PD-L1-specific mAb. P values shown, Wilcoxon matched-pairs test. In addition, statistical significance was not found comparing PD-L1 expression on BRAF wild-type versus mutant cell lines, either before or after IFN-γ exposure (Mann-Whitney test, p-values not shown).
Table 1

Correlation of PD-L1 expression by melanocytes with the presence of immune infiltrates is independent of BRAF mutational status.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Number of cases/total cases (%)</th>
<th>( p )</th>
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<tbody>
<tr>
<td></td>
<td>PD-L1+</td>
<td>PD-L1-</td>
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<tr>
<td></td>
<td>TIL+</td>
<td>TIL-</td>
<td>TIL+</td>
</tr>
<tr>
<td>All cases</td>
<td>52</td>
<td>20/21 (95)</td>
<td>1/21 (5)</td>
</tr>
<tr>
<td>BRAF V600E</td>
<td>22</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>No BRAF mutation</td>
<td>30</td>
<td>10/11 (91)</td>
<td>1/11 (9)</td>
</tr>
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\( a \) ≥5% of melanocytes with membranous PD-L1 expression

\( b \) including mild, moderate, and severe immune infiltrates (including TIL and associated histiocytes)

\( c \) Fisher’s exact test

\( d \) The relationship of melanocyte PD-L1 expression with TIL was previously reported for 21 of these cases (Ref 2).