PD-L1 Expression and Immune Escape in Melanoma Resistance to MAPK Inhibitors

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Abstract

Purpose: To examine the relationship between immune activity, PD-L1 expression, and tumor cell signaling, in metastatic melanomas prior to and during treatment with targeted MAPK inhibitors.

Experimental Design: Thirty-eight tumors from 17 patients treated with BRAF inhibitor (n = 12) or combination BRAF/MEK inhibitors (n = 5) with known PD-L1 expression were analyzed. mRNA expression arrays were performed on all pretreatment (PRE, n = 17), early during treatment (EDT, n = 8), and progression (PROG, n = 13) biopsies. HLA-A/HLA-DPB1 expression was assessed by IHC.

Results: Gene set enrichment analysis (GSEA) of PRE, EDT, and PROG melanomas revealed that transcriptome signatures indicative of immune cell activation were strongly positively correlated with PD-L1 staining. In contrast, MAPK signaling and canonical Wnt/β-catenin activity was negatively associated with PD-L1 melanoma expression. The expression of PD-L1 and immune activation signatures did not simply reflect the degree or type of immune cell infiltration, and was not sufficient for tumor response to MAPK inhibition.

Conclusions: PD-L1 expression correlates with immune cells and immune activity signatures in melanoma, but is not sufficient for tumor response to MAPK inhibition, as many PRE and PROG melanomas displayed both PD-L1 positivity and immune activation signatures. This confirms that immune escape is common in MAPK inhibitor–treated tumors. This has important implications for the selection of second-line immunotherapy because analysis of mechanisms of immune escape will likely be required to identify patients likely to respond to such therapies.

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Introduction

The MAPK pathway is constitutively activated in the majority of cutaneous melanomas (1), most commonly via mutations affecting BRAF kinase. Targeted inhibition of the MAPK pathway, with single-agent BRAF inhibitors or combined BRAF and MEK inhibitors, has improved the progression-free survival (PFS) and overall survival (OS) of patients with BRAFV600E mutant metastatic melanoma (2). However, only 20% of patients remain progression free in the long term (3), and the majority will develop resistance within 12–24 months of commencing treatment via mechanisms that reactivates MAPK signaling and/or enhance PI3K/AKT pathway activity (4–6). The genetic mechanisms of resistance to MAPK inhibitors are varied and heterogeneous. Nevertheless, in 20%–40% of patients who progress while receiving combination BRAF and MEK inhibitor therapy, the mechanism of resistance remains unknown (7, 8).

The immune system contributes to the antitumor activity of BRAF inhibitors (9). Inhibition of the MAPK pathway promotes a favorable immune microenvironment by increasing the expression of melanoma antigens, downregulating immunosuppressive cytokines and increasing the infiltration of CD4+ and CD8+ lymphocytes early during treatment (EDT; within 3–15 days of initiating therapy; refs. 10–13). Significantly, the density of the intratumoral CD8+ lymphocyte infiltrate correlates with reduction in tumor size (11) and an improved response to BRAF inhibition (14). Immune suppressive components within the melanoma microenvironment may also play an important role in BRAF inhibitor responses. The absence of the immunosuppressive programmed death receptor-ligand-1 (PD-L1) at baseline is associated with improved response to BRAF inhibition (14). We recently confirmed that tumor PD-L1 expression is significantly altered during MAPK inhibitor therapy. In patients with positive tumor PD-L1 staining in the sample taken prior to
Mechanisms of Resistance in MAPK Inhibitor–treated Melanoma

Translational Relevance

Targeted MAPK inhibitors have revolutionized the treatment of patients with advanced BRAF-mutant melanoma; however, the majority of patients develop resistance through various known and unknown (up to 40%) mechanisms. We identified that PD-L1 expression is associated with transcriptional signatures reflecting immune cell activation, regardless of the density and type of tumor immune cell infiltration. Our work confirms that mechanisms of immune escape are present in melanomas resistant to MAPK inhibition. These findings have important implications for the management of patients who acquire resistance to MAPK inhibitors for whom concurrent or sequential treatment with immunotherapies is being contemplated. Our results suggest that analysis of mechanisms of immune escape will be required to identify progressing MAPK inhibitor–treated metastatic melanoma patients who are likely to respond to subsequent anti-PD-1 immunotherapy.

Materials and Methods

Study design

Thirty-eight biopsies from 17 patients were included in the study; their clinical characteristics are summarized in Table 1. All patients had BRAFV600-mutant metastatic melanoma, had not received prior MAPK inhibitors or immunotherapy, and were treated with either a BRAF inhibitor (dabrafenib or vemurafenib) or a combination of BRAF and MEK inhibitors (dabrafenib and trametinib) as part of a clinical trial. Patients treated with BRAF inhibitor monotherapy were treated with either dabrafenib (150 mg twice daily or total daily dose of at least 300 mg daily) or vemurafenib (960 mg twice daily). The majority of patients treated with combination BRAF and MEK inhibitors received maximal recommended daily doses of dabrafenib (300 mg) plus trametinib (2 mg). Patient 13 received 150 mg/1.5 mg dabrafenib/trametinib. All 17 patients had a pretreatment (PRE) melanoma tissue sample obtained before commencing MAPK inhibitor therapy and a matched early during treatment (EDT) biopsy ($n = 8$), or a matched progressing (PROG) lesion removed ($n = 13$), a subset of which ($n = 4$) had both an EDT and PROG biopsy. All patients gave informed consent and all biopsies were conducted according to the Treat, Excise and Analyze for Melanoma (TEAM) protocol at Melanoma Institute Australia (X11-0289, HREC/11/RPAH/444). For all patients, formalin-fixed, paraffin-embedded (FFPE) biopsies had been previously described as part of a larger study describing the immune infiltrate and tumor PD-L1 expression using IHC (10).

Melanoma tissue samples

Fresh melanoma samples were macrodissected by a pathologist and enriched tumor portions were snap frozen. Frozen tumor sections were cut, stained with hematoxylin and eosin (H&E), and scored for the following parameters: % tumor cells, % necrosis, degree of pigmentation, predominant cell shape, and cell size of the most cellular portion of tumor. Tumor foci were carefully

Table 1. Patient, treatment outcome and resistance characteristics

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<th>Patient ID</th>
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<th>BRAF Mutation</th>
<th>MAPK Inhibitor</th>
<th>PRE PD-L1 (IHC)</th>
<th>EDT PD-L1 (IHC)</th>
<th>PROG PD-L1 (IHC)</th>
<th>RECIST Responsea</th>
<th>PFS, months</th>
<th>Time to death (days)</th>
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<th>Resistance Mechanismsb</th>
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Abbreviations: Dab, dabrafenib; EDT, early during treatment biopsy; ND, not determined (biopsy not available); PRE, pretreatment biopsy; PROG, progression biopsy; Tram, trametinib.

aPercent change from baseline sum of diameters.
bResistance mechanisms derived from (4, 8).

Mutation found in PRE and PROG.
macrodissected using the marked frozen section as a guide to meet minimum criteria for tumor cell content (>70%) and amount of necrosis (<30%), as described previously (1). High percentage tumor content was verified by examining post macrodissection frozen section slides. Total RNA was extracted from 10 to 20 mg of tumor. Tissue samples were homogenized using a high-speed agitation Polytron blender (Kinematica) in the presence of TRIzol (Life Technologies). Following homogenization, chloroform was added and the sample centrifuged. The top phase was removed and mixed with 70% ethanol. The RNA was then isolated and purified with an RNeasy purification kit with DNase I digestion on the column (Qiagen). The quality of the RNA preparations was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The extracted total RNA was of consistently high quality (RNA integrity number: 8–10).

Gene expression analysis
Gene expression analysis was performed on all samples taken at baseline (PRE, \(n = 17\)), early during treatment (EDT, \(n = 8\)) samples, and at progression (PROG, \(n = 13\)) from the 17 patients, using the Sentrix HumanHT-12 v4.0 Expression BeadChip (Illumina). Gene expression data were normalized using the LumiR method, as described previously (15). Rank ordering of gene expression data was carried out using the linear model for microarray module (LimmaGP) in GenePattern and analysis was performed using gene set enrichment analysis in preranked mode (GSEA pre-ranked; refs. 16, 17). Transcriptome analysis was also performed using single-sample gene set enrichment analysis (ssGSEA; ref. 18). This is a nonparametric, unsupervised method of gene set enrichment that generates an enrichment score representing the degree of absolute enrichment of a gene set for each tumor sample. The gene sets used in ssGSEA analysis consisted of release 5.2 of the Hallmark gene set (refined gene set that define specific biological processes; ref. 19) with a few additional immune (from the C2 Molecular Signature Database) and MAPK activation (20) signatures included. The correlations between immune cell markers and ssGSEA enrichment scores was then calculated using the Spearman correlation coefficient in the nearest neighbor algorithm within the Morpheus web based tool (https://software.broadinstitute.org/morpheus/). The microarray data has been submitted to Gene Expression Omnibus public database; accession code GSE99898.

IHC
All IHC staining was carried out on 4-μm thick sections using an Autostainer Plus (Dako; Agilent Technologies) with appropriate positive and negative controls. Sections were baked for 60 minutes at 60°C in a dehydration oven and heat-induced epitope retrieved in the PT link (Dako; Agilent technologies) using EnVision FLEX low pH (pH=6) target retrieval solution for 20 minutes at 97°C and then cooled to room temperature in TBST wash buffer for 5 minutes. Slides were incubated with the following antibodies at the following dilutions: HLA-A (Abcam; EP1393Y/ab52922) 1:400, HLA-DPB1 (Abcam; AB55152) 1:100. Antibody detection used the EnVision FLEX Kit (K8023) with a DAB chromagen for visualization according to the manufacturer’s instructions (Dako- Agilent technologies). Slides were then counterstained with hematoxylin. Assessment of tumor HLA-A and HLA-DPB1 expression was undertaken using an immune-reactive score (21) ranging from 0 to 300, made up of a four-tiered intensity score (0–3) multiplied by the percentage of tumor (0–100) expressing these molecules. All IHC slides were independently reviewed (by H. Kakavand and R.V. Rawson) blinded to the gene expression data.

Statistical analysis
Statistical analyses were conducted with "PASW Statistics 21" SPSS, IBM. The Wilcoxon matched-pairs method was used to test for significant changes in immune markers between the different biopsy time points (PRE, EDT, and PROG). Correlations between different immune markers were conducted using the Spearman ρ test. Categorical assessment of differences in markers was conducted using the Mann–Whitney U test. Statistical significance was defined as a probability level < 0.05.

Results
Patients and MAPK inhibitor therapies
We analyzed 38 fresh-frozen tumor samples derived from 17 patients treated with either a BRAF inhibitor alone (\(n = 12\)) or a combination of BRAF and MEK inhibitors (\(n = 5\)). Patients were segregated on the basis of tumor PD-L1 IHC expression. The clinicopathologic characteristics of the patients included in this study are summarized in Table 1. All patients had either the BRAFV600E (\(n = 9\)) or BRAFV600K (\(n = 8\)) mutation, and 8 patients (46%; 5 of 8 with V600E melanoma) had detectable tumor PD-L1 expression at baseline, as reported previously (10). There were no significant differences in clinical characteristics (i.e., age, sex, V600E/K mutation status) between the baseline tumor PD-L1 positive (\(n = 8\)) and tumor PD-L1 negative (\(n = 9\)) groups. All patients were naïve to immunotherapy and MAPK inhibitors at baseline.

IHC PD-L1 correlates
In this small dataset, there was no correlation between baseline PD-L1 or change in PD-L1 from baseline with RECIST response. We also examined PD-L1 expression and the tumor microenvironment and confirmed that tumor immune-reactivity for PD-L1 was weakly correlated with intratumoral and peritumoral tumor-infiltrating lymphocytes (intratumoral: \(r = 0.369, P = 0.018\)); peritumoral: \(r = 0.310, P = 0.049\)), CD4+ lymphocytes (intratumoral: \(r = 0.507, P < 0.01\)); peritumoral: \(r = 0.400, P = 0.010\)), CD8+ lymphocytes (intratumoral: \(r = 0.489, P < 0.01\)); and PD-L1 expression with tumor immune-reactivity for PD-L1 was strongly correlated with tumor-infiltrating lymphocytes (intratumoral: \(r = 0.570, P < 0.001\)); peritumoral: \(r = 0.517, P < 0.001\)) and tumor HLA-A expression (intratumoral: \(r = 0.629, P < 0.001\)); peritumoral: \(r = 0.570, P < 0.001\)).

Figure 1. Heatmap of IHC expression of tumor PD-L1 and immune cell subsets in PRE, EDT, and PROG melanomas. Spearman rank correlation coefficient between PD-L1 and the immune cells is shown.
peritumoral: \( r = 0.365, P = 0.021 \) and PD-1+ lymphocytes (intratumoral: \( r = 0.591, P < 0.01 \); peritumoral: \( r = 0.517, P < 0.01 \)) in the 38 melanoma biopsies analyzed in this study (i.e., \( n = 17 \) PRE, \( n = 8 \) EDT, and \( n = 13 \) PROG; Fig. 1). However, we noticed significant variation in the expression levels of PD-L1 and the density and distribution of immune cell infiltration (Fig. 1). For instance, high-density intratumoral and/or peritumoral lymphocytic infiltration was observed in melanomas with undetectable PD-L1 expression (patient 03 PRE and patient 08 EDT), low PD-L1 (patient 01 EDT and patient 10 PROG), and high PD-L1 tumor expression (patient 15 EDT and patient 16 EDT; Fig. 1).

### Tumor signaling and PD-L1 correlates

To explore signaling pathways associated with immune cell infiltration and tumor PD-L1 expression, we examined signaling activity in these melanoma biopsies using Illumina mRNA microarray data. We performed unbiased single-sample gene set enrichment analyses (ssGSEA), an extension of GSEA that defines an enrichment score of a gene set for each of the 17 PRE biopsies in our cohort (18). We sought to identify gene signatures that are most highly correlated with PD-L1 expression. Pathway activity indicative of immune cell activation including IFN-\( \gamma \) signatures and KEGG antigen-processing and presentation signatures were strongly positively correlated with PD-L1 staining (Fig. 2). The ‘KEGG antigen processing and presentation’ gene set includes 77 genes belonging to MHC class I and II pathways such as MHC molecules (e.g., HL-A-A, -B, -C, HL-A-DM, HL-A-DOA, HL-A-DRB1), chaperone and transporter proteins (e.g., calreticulin, TAP), peptidase involved in protein transport and processing within the endoplasmic reticulum (e.g., HSPA-2, 4, 5, 6, 8).

We also found that MAPK signaling and canonical Wnt/\( \beta \)-catenin activity were negatively associated with PD-L1 protein expression in the 17 PRE tumors (Fig. 2). Significantly, PD-L1 expression in PRE biopsies correlated with immune signaling activity (i.e., IFN signaling and antigen-processing gene sets) irrespective of the degree of intratumoral or peritumoral immune cell infiltration. For instance, the PRE biopsies of patients 06 and 16 expressed PD-L1 and IFN signatures but only the PRE tumor derived from patient 06 showed significant lymphocytic infiltration (Fig. 2). Thus, immune cell activity genes sets, such as Hallmark IFN\( \gamma \) and \( \alpha \) signatures, were significantly correlated with PD-L1 expression rather than any particular immune cell subset (Fig. 2).

We next explored eight patients with matched PRE and EDT biopsies; 7 of the 8 EDT tumors (i.e., all but patient 17) showed a dramatic increase in lymphocytic infiltration from PRE to EDT, including increasing CD4+ and CD8+, and PD-1+ immune cell populations (Fig. 3A). Irrespective of the substantial changes in immune cell infiltration, PD-L1 expression showed minimal changes from PRE to EDT in all patients, except patient 16 (Fig. 3A). Consequently, the immune cell infiltration early on therapy did not correlate with PD-L1 expression (intratumoral TILs: \( r = 0.432, \) NS; Fig. 3A). Preranked gene set enrichment analysis comparing the PRE and EDT tumor groups identified 23 gene sets upregulated and 16 downregulated (\( q < 0.1 \)) in the EDT tumors compared with the PRE melanomas (Supplementary Table S1). We noted that pathway activity indicative of immune cell activation, including type I IFN signatures, antigen presentation signatures as well as epithelial–mesenchymal and invasive gene sets, which also correlate with immune modifying factors, such as interleukins and chemokines (22) were significantly upregulated in the EDT tumors. In contrast, gene sets indicative of proliferation (E2F, MHC, and MAPK signaling were downregulated in EDT tumors compared with the PRE melanomas (Supplementary Table S1). Importantly, the immune activity signatures were very strongly correlated with PD-L1 positivity and moderately correlated with intratumoral CD4+ and CD8+ immune cells (Fig. 3B). This is clearly demonstrated in patients 10 and 17; patient 10 showed immune cell infiltration early during therapy but no associated increase in PD-L1 or immune activation signatures. In contrast, patient 17 showed a slight increase in PD-L1 expression from PRE to EDT, no concomitant increase in intracellular immune cells, but a dramatic increase in immune cell activation gene sets (Fig. 3B).

There were 13 patients with matched PRE and PROG biopsies for analysis and nine PROG tumors showed immune cell infiltration early from PRE to PROG and this change did not reflect PD-L1 expression changes (Fig. 4). The lack of correlation between PD-L1 and immune cell infiltrates from PRE to PROG, presumably reflects an absence of immune cell activation. This was confirmed by examining RNA sequence changes in the PRE versus PROG tumors using gene set enrichment analysis (Fig. 4). In contrast to EDT biopsies, few immune activation gene sets were significantly upregulated in the PROG tumors (\( q < 0.1 \); Supplementary Table S2). We also explored whether resistance effectors were associated with specific profiles of immune cell activity and infiltration derived from previous work (4, 8). Although, the small numbers of diverse resistance mechanisms did not permit for correlation analysis, we did confirm that MAPK activity signatures were restored in most PROG tumors and this reflected the presence of MAPK-activating mutations, including MEK1, MEK2, BRAF, and N-RAS alteration (Fig. 4; Table 1). Moreover, MAPK signaling in PRE and PROG tumors was strongly correlated with loss of intratumoral PD-
L1 expression, CD4⁺ cells, CD8⁺ cells, and the KEGG antigen processing and presentation signature (PD-L1: \( r = -0.5192, P < 0.01; \) CD4⁺: \( r = -0.4827, P = 0.01; \) CD8⁺: \( r = -0.4338, P = 0.03; \) KEGG_processing \( r = -0.6429, P = 0.02; \) Fig. 4).

**Protein validation of antigen presentation signatures**

"Interferon response" and "KEGG antigen processing and presentation" signatures were strongly associated with PD-L1 expression in the melanomas assessed and these gene sets include the Pratilas_MAPK_UP signature derived from ref. 20.

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**Figure 3.**

A. Comparison of the change in IHC expression from PRE to EDT of PD-L1 and immune cell subsets. Spearman's rank correlation coefficient between change in PD-L1 and the change in immune subsets is shown. B. Heatmap showing tumor PD-L1, immune cell subsets, and correlated transcriptome signatures (ssGSEA scores) in 8 PRE-EDT paired melanomas. Spearman's rank correlation coefficient between PD-L1 and the transcriptome signatures is shown. Pratilas_MAPK_UP signature derived from ref. 20.

**Figure 4.**

A. Comparison of the change in IHC expression from PRE to PROG of PD-L1 and immune cell subsets. Spearman rank correlation coefficient between change in PD-L1 and the change in immune subsets is shown. B. Heatmap showing tumor PD-L1, immune cell subsets, and selected transcriptome signatures (ssGSEA scores) in 13 PRE-PROG paired melanomas. Spearman's rank correlation coefficient between PD-L1 and the transcriptome signatures is shown. Pratilas_MAPK_UP signature derived from ref. 20.
HLA-A and HLA-DPB1 molecules. In fact, the expression of these individual genes was also correlated with the PD-L1 immunoscore in these 38 tumors (HLA-A: \( r = 0.502, P = 0.001; \) HLA-DPB1: \( r = 0.595, P < 0.001 \)). We therefore carried out IHC analysis of these markers in the matched FFPE blocks of 36 of 38 fresh frozen biopsies. The expressions of the tumor HLA-A (Supplementary Fig. S1A–S1D) and HLA-DPB1 molecules were restricted to cytoplasmic membrane staining. In the 36 samples taken at various time points of treatment, the expression of tumor PD-L1 significantly positively correlated with the expression of tumor HLA-A (\( r = 0.48, P = 0.004 \)), but not with tumor HLA-DPB1 (\( P > 0.05 \)). The RNA expression for all available HLA genes in the tumor PD-L1-positive and negative groups was compared with the IHC expression of HLA-A and HLA-DPB1 (Supplementary Fig. S2A). The change in expression of the HLA genes from PRE to EDT (Supplementary Fig. S2B) and from PRE to PROG (Supplementary Fig. S2C) was also compared with the change in IHC expression of HLA-A and HLA-DPB1. This comparison showed that there was significantly increased tumor HLA-A expression in tumor PD-L1-positive specimens compared with negative samples (\( P = 0.0005 \)). There was also a trend toward a positive correlation between change in (\( \Delta \)) tumor PD-L1 by IHC from PRE to EDT and PRE to PROG with change in (\( \Delta \)) HLA gene expression from PRE to EDT and PRE to PROG, respectively.

**Discussion**

Identifying resistance mechanisms to targeted MAPK inhibitors has largely focused on intracellular pathway analyses based on genomic and transcriptomic data (4–6, 8, 23) with limited focus on the immune microenvironment of the tumor. Previous characterization, by our group, of the immune infiltrate of tumors taken at multiple time points from patients treated with MAPK inhibitors revealed an abundance of tumor-infiltrating lymphocytes early during treatment as well as significant differences in tumor PD-L1 expression at progression, which was dependent on the tumor PD-L1 expression at baseline (10). In patients with positive tumor PD-L1 staining at baseline, there was a significant decrease in tumor PD-L1 expression in the progression sample, whereas those that were tumor PD-L1 negative at baseline, there was a significant increase in tumor PD-L1 expression in the progression samples. These results presented a scientific rationale for combinations of MAPK inhibitors and immunotherapies (24), which are currently being assessed in clinical trials (25, 26) and also provide a basis to explore response to subsequent anti-PD-L1-based therapies for patients whose disease develops resistance to MAPK inhibitors and progresses. A proportion of our patients had gene expression data available for analysis on matched fresh frozen tissue, which provided us with a unique opportunity to assess pathways and immune profiles associated with response and resistance in MAPK inhibitor–treated patients. In the current study, we have expanded on previous work demonstrating loss of antigen expression and depletion of CD8+ lymphocytes in the PROG biopsies of patients treated with MAPK inhibitors (27).

Tumor PD-L1 expression was positive in over half of the samples in this cohort (22/38 PD-L1+). We applied a nearest neighbor approach to predict gene expression signatures correlating with PD-L1 positivity in PRE, EDT, and PROG melanomas. Tumor PD-L1–positive samples showed enrichment of immune activation and antigen presentation gene sets, which has been shown previously in untreated stage III melanoma patient samples (28) and by IHC analysis (29). The association between PD-L1 expression and immune activation transcriptome signatures was most evident in the EDT biopsies analyzed here. Importantly, PD-L1 positivity was strongly associated with immune activation, rather than simply the degree and type of immune cell infiltrate. This may reflect the potential role of other cell types, including natural killer cells, in regulating tumor-associated PD-L1 accumulation and further studies are required to elucidate this potential link. We also found that PD-L1 expression correlated with loss of MAPK and \( \beta \)-catenin activation signatures. This supports previous data showing that MAPK inhibition in BRAPV600E-mutant melanoma promotes immune cell infiltration and activation (11, 30) and \( \beta \)-catenin activity prevents T-cell priming against tumor neoantigens (31).

Our work confirms that PD-L1 expression is associated with immune activity gene signatures in melanoma, but this was not sufficient for tumor response, as many PRE and PROG melanomas displayed both PD-L1 positivity and immune signature activation with or without significant immune cell infiltration. This strongly suggests that mechanisms of immune escape are present in these tumors, and this may include alterations in IFN signaling pathways and loss of antigen presentation machinery (32). For instance, the “KEGG antigen processing and presentation” signature was strongly negatively associated with MAPK reactivation in PROG tumors and although HLA-A protein induction correlated with PD-L1 upregulation in our melanoma samples, HLA-A was not always upregulated along with PD-L1 and immune activation signatures (e.g., patient 09 PROG and patient 15 PROG). This raises the possibility that lack of HLA upregulation in tumor cells, may potentiate tumor cell escape from the immune system (33). The loss of antigen presentation machinery in tumors resistant to MAPK inhibitors could potentially decrease the efficacy of subsequent second-line immune-checkpoint inhibitor therapies. Significantly, the use of IFN monotherapy or in combination with a MEK1/2 inhibitor was able to restore antigenicity and immune infiltration in papillary thyroid cancer cell line models (34) and could present another form of combination therapy for patients having acquired resistance to MAPK inhibitors.

There are currently several clinical trials in progress that are assessing the efficacy of different combinations of targeted and immune therapies in metastatic melanoma patients (25, 26). In retrospective assessment of patients treated as part of clinical trials of PD-1 inhibitors, the positive expression of tumor PD-L1 was shown to be associated with significantly higher response rates (35, 36). PD-L1 positivity is also associated with immune activity in MAPK inhibitor–treated patients. Nevertheless, the low levels of PD-L1 expression and the heterogeneity of PD-L1 expression complicates triaging patients into subsequent treatment options once they have progressed on MAPK inhibitors. Furthermore, the presence of infiltrating immune cells, immune cell signaling, and PD-L1 expression in a subset of PRE and PROG melanomas suggests that immune escape is not uncommon and this has significant implications for the identification of patients likely to respond to second-line immunotherapies.
Disclosures of Potential Conflicts of Interest

A.M. Menzies reports receiving speakers bureau honoraria from Bristol-Myers Squibb and Roche, and is a consultant/advisory board member for Chugai, MSD, Novartis, and Pierre-Fabre. M.S. Carlino is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, MSD, and Novartis. R.F. Kefford is a consultant/advisory board member for Bristol-Myers Squibb, Merck, and Novartis. R.P.M. Saw reports receiving speakers bureau honoraria from Bristol-Myers Squibb. J.F. Thompson is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, and Protecuve. G.V. Long is a consultant/advisory board member for Bristol-Myers Squibb, Merck MSD, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

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