BRAF Inhibition Is Associated with Enhanced Melanoma Antigen Expression and a More Favorable Tumor Microenvironment in Patients with Metastatic Melanoma

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Abstract

Purpose: To evaluate the effects of BRAF inhibition on the tumor microenvironment in patients with metastatic melanoma.

Experimental Design: Thirty-five biopsies were collected from 16 patients with metastatic melanoma pretreatment (day 0) and at 10 to 14 days after initiation of treatment with either BRAF inhibitor alone (vemurafenib) or BRAF + MEK inhibition (dabrafenib + trametinib) and were also taken at time of progression. Biopsies were analyzed for melanoma antigens, T-cell markers, and immunomodulatory cytokines.

Results: Treatment with either BRAF inhibitor alone or BRAF + MEK inhibitor was associated with an increased expression of melanoma antigens and an increase in CD8+ T-cell infiltrate. This was also associated with a decrease in immunosuppressive cytokines [interleukin (IL)-6 and IL-8] and an increase in markers of T-cell cytotoxicity. Interestingly, expression of exhaustion markers TIM-3 and PD1 and the immunosuppressive ligand PDL1 was increased on treatment. A decrease in melanoma antigen expression and CD8 T-cell infiltrate was noted at time of progression on BRAF inhibitor alone and was reversed with combined BRAF and MEK inhibition.

Conclusions: Together, these data suggest that treatment with BRAF inhibition enhances melanoma antigen expression and facilitates T-cell cytotoxicity and a more favorable tumor microenvironment, providing support for potential synergy of BRAF-targeted therapy and immunotherapy. Interestingly, markers of T-cell exhaustion and the immunosuppressive ligand PDL1 are also increased with BRAF inhibition, further implying that immune checkpoint blockade may be critical in augmenting responses to BRAF-targeted therapy in patients with melanoma. Clin Cancer Res; 19(5); 1225–31. ©2013 AACR.

Introduction

Melanoma remains a major world health problem (1, 2), although recent advances in targeted therapy against oncogenic BRAF for melanoma have shown some promising results (3). Somatic mutations in the BRAF oncogene occur in more than half of melanomas, with the vast majority of these harboring an activating point mutation (V600E; ref. 3). This oncogenic mutation leads to constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway and increased oncogenic potential through a variety of mechanisms including reduced apoptosis, increased invasiveness, and increased metastatic behavior (4). Recent in vitro data also suggest that BRAF(V600E) could also contribute to immune escape (5).

Targeted therapy against oncogenic BRAF for metastatic melanoma results in objective responses in the majority of patients whose tumors harbor BRAF(V600E) (6). Despite this, resistance to therapy remains a significant issue, with a median duration of response between 6 and 7 months (6). There is a great deal of ongoing research to determine mechanisms of resistance and strategies to overcome resistance (7–9). Multiple distinct mechanisms of resistance have already been identified in recent months (10–13).

Combination of BRAF-targeted therapy with other signal transduction inhibitors has been proposed on the basis of evidence that other pathways become activated upon
This study analyzes immune responses in serial tumor biopsies of patients with metastatic melanoma treated with BRAF-targeted therapy. Significantly, we show that BRAF inhibition is associated with an increase in melanoma antigen expression and T-cell infiltrate and a decrease in immunosuppressive cytokines in tumors of treated patients. An increase in markers of T-cell cytotoxicity was also noted. Interestingly, both melanoma antigen expression and T-cell infiltrate were abrogated at disease progression. Paradoxically, BRAF inhibition was associated with an increase in T-cell exhaustion markers TIM-3 and PD1 and the immunosuppressive ligand PDL1. Together, these data support the hypothesis that combined BRAF-targeted therapy and immunotherapy may improve responses in patients with metastatic melanoma. This work provides the basis for future research studies and clinical trials that will focus on understanding the complex interplay between the tumor, immune system, and the tumor microenvironment in response to targeted therapy.

Translational Relevance
This study analyzes immune responses in serial tumor biopsies of patients with metastatic melanoma treated with BRAF-targeted therapy. Significantly, we show that BRAF inhibition is associated with an increase in melanoma antigen expression and T-cell infiltrate and a decrease in immunosuppressive cytokines in tumors of treated patients. An increase in markers of T-cell cytotoxicity was also noted. Interestingly, both melanoma antigen expression and T-cell infiltrate were abrogated at disease progression. Paradoxically, BRAF inhibition was associated with an increase in T-cell exhaustion markers TIM-3 and PD1 and the immunosuppressive ligand PDL1. Together, these data support the hypothesis that combined BRAF-targeted therapy and immunotherapy may improve responses in patients with metastatic melanoma. This work provides the basis for future research studies and clinical trials that will focus on understanding the complex interplay between the tumor, immune system, and the tumor microenvironment in response to targeted therapy.

Patient samples
Patients with metastatic melanoma containing BRAF V600E mutation (confirmed by genotyping) were enrolled on clinical trials for treatment with a BRAF inhibitor (vemurafenib) or combined BRAF + MEK inhibitor (dabrafenib + trametinib; Supplementary Table S1) and were consented for tissue acquisition per Institutional Review Board (IRB)-approved protocol. Tumor biopsies were conducted pre-treatment (day 0), at 10 to 14 days on treatment, and/or at time of progression if applicable. Formalin-fixed tissue was analyzed to confirm that viable tumor was present via hematoxylin and eosin (H&E) staining. Additional tissue was snap-frozen and stored in liquid nitrogen or was immediately processed for purification of RNA.

Purification of total RNA
Samples were homogenized and disrupted using a mortar and pestle followed by use of a QIAshredder. A QIACube was used to harvest RNA using the RNeasy Mini Protocol (Qiagen).

Quantitative PCR
Total RNA (250 ng) was used as template, and Superscript VILO cDNA Synthesis Kit (Invitrogen) was used to generate cDNA. Quantitative real-time PCR was carried out on an Applied Biosystems 7300 machine.

Immunohistochemistry
Tumor biopsies were stained with primary antibodies for MART-1 (Covance, SIG-38160–1000), HMB-45(gp100) (Leica, PA0027), CD4 (Leica, NCL-CD4–1F6), CD8 (Leica. PA0183), Perforin (Santa Cruz, sc-374346), TIM-3 (R&D Systems, AF2365), PDL1 (LS-Bio, LS-B3368), or Granzyme B (Abcam, ab4059) followed by a secondary antibody for horseradish peroxidase and then 3,3′,5′-diaminobenzidine (DAB) or blue chromagen. Stained slides were interpreted by a dedicated dermatopathologist.

Counting of CD8+ T cells
CD8+ T-cell count was conducted on slides from pre-treatment and on-treatment tumor biopsies in 4 adjacent high-power fields (HPF) in the areas of highest density of CD8-positive cells. Only positive signals with clear lymphocyte morphology were evaluated.

Immunofluorescence
Sections from formalin-fixed, paraffin-embedded (FFPE) melanoma tumor biopsies were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked in 3% hydrogen peroxide in water. After rinsing, heat-induced antigen retrieval was conducted. Nonspecific binding was blocked by 20% serum blocker and endogenous avidin + biotin blocking system. Primary antibody (monoclonal antibody targeting MART-1) and fluorescein isothiocyanate (FITC)-conjugated secondary antibody were then applied. Images were acquired using a Nikon Eclipse-80i fluorescence microscope.

Statistics
Statistics were conducted using GraphPad Prism or the R-statistical package.

Results
BRAF inhibition is associated with increased melanoma antigen expression in tumors of patients with metastatic melanoma
Given prior preclinical findings that BRAF inhibition leads to increased expression of melanoma antigens in melanoma cell lines and fresh tumor digests in vitro, we...
sought to validate these findings in patients with metastatic melanoma undergoing treatment with a BRAF inhibitor (patients 1–5) or combined BRAF + MEK inhibitor (patients 6–18; Supplementary Table S1). We observed a significant increase in mRNA levels for melanoma antigens assayed after treatment with BRAF inhibitor (Fig. 1A). Melanoma antigen expression increased by 4.9-, 16.4-, 13.3-, and 14.1-fold in MART, TYRP-1, TYRP-2, and GP100 respectively. Of note, there was no statistically significant difference in melanoma antigen expression in biopsies from patients receiving a BRAF inhibitor alone versus combined BRAF + MEK inhibition ($P > 0.1$) with the exception of TYRP-2 ($P < 0.04$) which was higher in those treated with combined BRAF + MEK inhibition (data not shown). Findings of increased MART-1 expression were validated via staining of MART-1 protein using both immunohistochemistry (Fig. 1B; 40 magnification) and immunofluorescence (Fig. 1C) staining for the melanoma antigen MART-1 in pretreatment and on-treatment biopsies (patient 2) was conducted to confirm that protein expression correlated with mRNA expression. All microscopy was repeated at least 3 times with representative examples shown. P values are from a 2-tailed Student t test with a $\mu$ of 1, which corresponds to no change in mRNA levels on treatment. *, $P < 0.05$.

**BRAF Inhibition Facilitates a More Favorable Tumor Microenvironment**

Next, we conducted studies to test the ability of BRAF inhibition to augment T-cell infiltrate based on our preclinical data showing that increased MDA expression is associated with increased recognition by antigen-specific T cells. Tumor biopsies from patients with metastatic melanoma undergoing treatment with a BRAF inhibitor were stained by H&E and using immunohistochemistry for CD8-positive cells. We observed a significant increase in CD8+ T-cell infiltrate by immunohistochemistry in 10 of 11 patients (Fig. 2A–C). There was no difference in CD4+ T-cell infiltrate (data not shown).

**BRAF inhibition is associated with decreased immunosuppressive cytokines and markers of T-cell cytotoxicity but increased T-cell exhaustion markers and PDL1 in tumors of patients with metastatic melanoma**

Next, we analyzed the tumors of treated patients for expression of immunosuppressive cytokines, markers of T-cell cytotoxicity, T-cell exhaustion markers, and the immunosuppressive ligand PDL1 to determine the effects of BRAF inhibition on the tumor microenvironment. BRAF inhibition was associated with a significant decrease in the expression of immunosuppressive cytokines interleukin (IL)-6 and IL-8, whereas there was no significant change in TGF-β and IL-10 (Fig. 3A). A significant increase in...
expression was also observed in markers of T-cell cytotoxicity (perforin, granzyme B; Fig. 3B). Of note, T-cell exhaustion markers TIM-3 and PD1 were also significantly increased after BRAF inhibition (Fig. 3B). Expression of the immunosuppressive ligand PDL1 was also significantly increased following treatment with a BRAF inhibitor (Fig. 3C). These were validated using immunohistochemistry for markers with available antibodies (Fig. 3D and E). Of note, there was no change in HLA expression (Supplementary Fig. S2).

Melanoma antigen expression and CD8+ T-cell infiltrate are decreased at time of progression and restored through MEK inhibition

Tumor biopsies from patients with metastatic melanoma undergoing treatment with a BRAF inhibitor who progressed on therapy were assayed for melanoma antigen expression and CD8+ T-cell infiltrate as described previously. At the time of progression, there was a significant decrease in melanoma antigen expression (Fig. 4A and Supplementary Fig. S3) and CD8+ T-cell infiltrate. One of these patients was later enrolled on a combination trial incorporating combined BRAF + MEK inhibition (dabrafenib + trametinib) after progressing on BRAF monotherapy. A tumor biopsy conducted after initiation of combined BRAF + MEK therapy showed restoration of melanoma antigen expression and CD8+ T-cell infiltrate (Fig. 4A–C).

Discussion

The advent of therapy with BRAF inhibitors has produced remarkable clinical success (6) and brought new hope for patients with metastatic melanoma. However, the impressive response rates have been tempered by a short duration of response in the majority of patients (6). There is an intense effort underway to better understand mechanisms of resistance to BRAF-targeted therapy (7–9, 13), with several strategies proposed to counter this resistance. Combination with other targeted agents (14) has been proposed and clinical trials are currently underway. The addition of further MAPK blockade via use of an MEK inhibitor with a BRAF inhibitor extends the median duration of response from 5.6 to 9.5 months, although virtually all patients progress on therapy with very few complete responses noted (17).

Clinical studies have also shown striking successes for immunotherapy approaches in melanoma (18–20). Adoptive therapy, vaccines, immunomodulatory approaches, and immune checkpoint/tolerance blockades have all exhibited promising results, with ipilimumab now U.S. Food and Drug Administration (FDA)-approved on the basis of positive phase III clinical trial results in advanced melanoma (18) and other immune checkpoint inhibitors in clinical trials.

There is growing evidence that BRAF-targeted therapy may synergize well with immunotherapy. Prior in vitro
findings showed that pharmacologic manipulation of the MAPK pathway results in increased melanoma antigen expression in melanoma cells and this increase was associated with enhanced sensitivity to antigen-specific T cells (5).

More recently, our group and others have provided supporting evidence for these in vitro findings, showing an increase in tumor infiltrating lymphocytes in patients with metastatic melanoma treated with BRAF inhibitors (15, 16). An increase in immune infiltrate was seen in virtually all patients although the response to treatment was variable, raising the question as to whether the increase in T-cell infiltrate is directly related to MAPK pathway blockade or if it is secondary to tumor necrosis. The results in Fig. 4 showing restored T-cell infiltrate with combination BRAF + MEK would suggest that the increased T-cell infiltrate is more likely due to MAPK pathway inhibition, although further studies are necessary to better understand the response.

This article provides strong in vivo support but more importantly shows novel findings showing that BRAF inhibition is associated with increased melanoma antigen expression, increased markers of T-cell cytotoxicity, and decreased expression of immunosuppressive cytokines—all enhancing the tumor microenvironment. However, further characterization of the immune infiltrate reveals that the infiltrating T cells show an exhausted phenotype (with increased TIM-3 and PD1) and increased expression of the immunosuppressive ligand PDL1. These results suggest that BRAF inhibition promotes T-cell infiltration and increased melanoma antigen expression; however, the immune response may be limited due to an increase in exhaustion markers on T cells and an increase in PDL1. These findings are novel and have important clinical implications and may imply that successful combination therapy may require immune checkpoint blockade to enhance antitumor immunity.

Another novel finding in these studies was the observation that the increase in melanoma antigen expression and CD8+ T-cell infiltrate was abrogated at the time of progression. While intriguing, these findings need to be validated in a larger cohort of patients. The patterns suggest that reactivation of the MAPK pathway is responsible for suppression of melanoma antigens and re-establishment of an immunosuppressive tumor microenvironment. This hypothesis is further supported by our findings that subsequent MAPK pathway inhibition using an MEK inhibitor can restore melanoma antigen expression and promote infiltration of CD8+ T cells. It is unclear whether the use of a different BRAF inhibitor (dabrafenib) or the addition of the MEK inhibitor contributed to this phenomenon, although the recent literature would suggest that it is more likely to be the latter (21). Of note, in our in vitro studies leading up to these clinical findings (5) observed a deleterious effect of MEK inhibition on T lymphocytes which
raised the potential concern that MEK inhibition in patients may alter T-cell function. However in the present study, we saw no significant difference in T-cell infiltrate in patients receiving BRAF inhibitor monotherapy versus BRAF inhibitor plus MEK inhibitor. Functional studies on these T cells were not conducted in these patients; however, these initial data about T-cell infiltrate might suggest that MEK inhibition does not significantly impact T-cell function.

On the basis of the data presented herein, one could speculate that augmenting the immune response by providing pro-immune cytokines (such as IL-2) or immune checkpoint inhibitors (such as monoclonal antibodies targeting CTLA-4, PD1, or PDL1) will act synergistically with the immune infiltrate elicited by BRAF inhibition in patients with metastatic melanoma. Interestingly, Hooijkaas and colleagues showed that anti-CTLA-4 mAb in combination with BRAF inhibition did not enhance tumor growth control in an inducible murine model; however, this model also showed a decrease in T-cell infiltrate after BRAF inhibition which is contrary to what we see in patients (22). In a mouse model of BRAFV600E melanoma, Koya and colleagues showed improved antitumor activity, in vivo cytotoxic activity, and intratumoral cytokine secretion by adoptively transferred cells in combination with a BRAF inhibitor (23). Trials combining BRAF-targeted therapy and immunotherapy in patients are currently underway, although no data is currently available about response rates, duration of response, or findings from correlative studies. Coupled with these preclinical studies, our present findings reinforce the rationale for combined BRAF-targeted therapy and immunotherapy in the treatment of metastatic melanoma.

Future studies to further evaluate the immune response during BRAF inhibition are still needed. A deeper understanding of how these treatment modalities interact with each other will allow for optimal design of clinical trials to maximize response rates and duration of response in patients with metastatic melanoma.

Disclosure of Potential Conflicts of Interest

F.S. Hodi has served as a non-paid consultant to Genentech-Roche and received clinical trial support from Genentech. K.T. Flaherty has served as a consultant to GlaxoSmithKline and Roche/Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Piris, A. Boni, P. Hwu, K.T. Flaherty, D.E. Fisher, J.A. Wargo

Figure 4. Melanoma antigen expression and CD8+ T-cell infiltrate are decreased at time of progression and restored through MEK inhibition. A, tumors were harvested at time of progression and at time of treatment with combined BRAF inhibition and MEK inhibition for patient 3. mRNA levels of the melanoma antigens gp100, MART-1, TYRP-1, and TYRP-2 were assayed. B, immunohistochemistry (IHC) was conducted for CD8+ T cells on patient tumor samples. C, CD8+ T cells were identified and counted by a dedicated pathologist. Average CD8+ T-cell counts are plotted with error bars representing the SD of 4 measurements. *, P < 0.05.
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References

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