Selective BRAF Inhibitors Induce Marked T-cell Infiltration into Human Metastatic Melanoma

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Abstract

**Purpose:** To evaluate the effects of treatment with the potent mutant BRAF inhibitors GSK2118436 or vemurafenib (PLX4720) on immune responses to metastatic melanoma in tissues taken before and after treatment.

**Experimental Design:** Thirty-seven tumor biopsies were collected from 15 patients with unresectable American Joint Committee on Cancer stage III or IV melanoma immediately before and approximately 7 days after the commencement of BRAF inhibitor treatment and at the time of tumor progression. Immunohistochemical staining was carried out on the biopsies using specific antibodies for CD8, CD4, CD20, CD1a, and Granzyme B.

**Results:** Tumor infiltration by CD4+ and CD8+ lymphocytes increased markedly following BRAF inhibitor treatment (both \( p = 0.015 \)). There was a correlation between the degree of tumor infiltration by CD8+ and Granzyme B–expressing lymphocytes in post–BRAF inhibitor–treated biopsies (\( r = 0.690 \) and \( p = 0.013 \)). Increased intratumoral CD8+ lymphocyte expression was correlated with a reduction in tumor size and an increase in necrosis in posttreatment biopsies (\( r = 0.793, p = 0.011 \); and \( r = 0.761, p = 0.004 \), respectively).

**Conclusions:** The increase in tumor-infiltrating lymphocytes induced by treatment with BRAF inhibitors provides strong support for conducting trials that combine BRAF inhibitors with immunotherapy in the hope of prolonging clinical responses. Clin Cancer Res; 18(5); 1386–94. ©2011 AACR.

Introduction

The 1-year survival rate for patients with American Joint Committee on Cancer (AJCC) stage IV visceral metastatic melanoma is 33% (1). Until recently, the treatment options for patients with advanced-stage metastatic melanoma have proved largely ineffective in improving overall survival (2, 3). Mutation of the BRAF gene is present in approximately 50% of cutaneous melanomas and results in the constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, responsible for controlling cellular proliferation, apoptosis, and migration (4, 5). The activating mutations are V600E in 74% and V600K in 19% of melanomas in Australians (4). Clinical trials of the potent inhibitors of V600 mutant BRAF, vemurafenib (6, 7) and GSK2118436 (8, 9), show tumor shrinkage in the majority of patients with BRAF mutant metastatic melanoma. However, drug resistance is a limiting factor in efficacy with progression-free survival restricted to 5 to 7 months. A better understanding of the range of effects that BRAF inhibitors have on melanoma biology may provide additional therapies that can be combined with the BRAF inhibitor to improve melanoma treatment. The immune response to BRAF inhibitor treatment is one exciting new area open to exploitation.

During histopathologic examination of tissue biopsies from patients with metastatic melanoma who are receiving BRAF inhibitors, we observed an increase in the density of tumor-infiltrating lymphocytes (TIL) within and around the metastases in biopsies taken early after commencement of treatment (3–15 days; ref. 10). In vitro experiments on mutant BRAF melanoma cell lines suggested that this may reflect inhibition of the release of immunosuppressive cytokines such as interleukin (IL)-10, VEGF, and IL-6 (11), or reflect increased melanoma cell antigen presentation, resulting in increased T-cell recognition of the tumor cells.
**Translational Relevance**

This novel and important translational study of patients with metastatic melanoma analyzed the immune response in a unique cohort of biopsies collected before, within 7 days after, and at the time of tumor progression (N = 37 biopsies) following treatment with a highly active and potent BRAF inhibitor. Most significantly, we showed that T lymphocytes infiltrated tumors early following treatment with a BRAF inhibitor. With the recent publications showing, for the first time in metastatic melanoma, survival advantages for patients with melanoma treated with the immunotherapeutic agent ipilimumab and the BRAF inhibitor vemurafenib, our study is the first to provide a scientific rationale for the combination of these agents in human patients. Numerous future research studies and clinical trials will focus on the combination of BRAF inhibition and immunotherapy. Therefore, this study will provide essential information for researchers and clinicians alike in designing and conducting these trials.

(12), or enhanced Melan-A/MART-1 expression in melanoma cells, thereby increasing cytotoxic T-cell recognition (13). *In vitro* studies show that the BRAF inhibitor vemurafenib has little effect on T-cell viability and function at therapeutic concentrations (14). These studies led us to investigate changes in the degree and type of TILs in response to BRAF inhibitor treatment. In this study, we used immunohistochemical staining of the markers for CD4 (Th), CD8 (cytotoxic T cell), CD20 (B cell), CD1a (dendritic cells), and Granzyme B (cytotoxic T cells and natural killer cells) to examine the type, location, and density of TILs in response to BRAF inhibitor treatment.

**Materials and Methods**

**Study design**

Thirty-seven tumor samples were collected from 15 patients with metastatic melanoma treated with a BRAF inhibitor as part of phase I and II clinical trials that took place from 2009 to 2011. The BRAF mutation status was determined as previously described (Table 1; ref. 4). Patients eligible for this study had unresectable AJCC stage III or stage IV BRAF mutant melanoma and were treated with either GSK2118436 or vemurafenib BRAF inhibitors at the dosages and durations described in Table 1. Biopsied tumor specimens were collected from consenting patients within 7 days prior to BRAF inhibitor treatment in all but 2 cases (PRE), 3 to 15 days post-BRAF inhibitor (POST), and at disease progression (PROGRESSION) as part of the Treat Excise Analyze for Melanoma (TEAM) study at the Melanoma Institute in Sydney, as approved by the Royal Prince Alfred Hospital Research Ethics Committee Protocol No. X10-0305 and HREC/10/ RPAH. Clinical and follow-up details were collated on all patients (Table 1). The size of the biopsied tumor was measured with calipers before and after BRAF inhibitor treatment. 2[18F]Fluoro-2-deoxy-D-glucose positron emission tomographic (FDG-PET) and computed tomographic (CT) staging was carried out on patients to evaluate clinical responses. The CT response was defined by the criteria set out by the Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 for GSK2118436 and RECIST 1.1 for vemurafenib (15, 16). The PET response was evaluated with the maximum standardized uptake value (SUVmax).

**Immunohistochemistry**

The biopsies were fixed in 10% buffered formaldehyde. After overnight fixation, they were embedded in paraffin wax and 4-μm thick sections were cut. All immunohistochemical staining was carried out on a Leica Bond-Max autostainer (Leica Microsystems) according to the manufacturer’s protocol, with appropriate positive and negative controls. Sections were baked at 60°C for 60 minutes in a dehydration oven, then dewaxed in Bond Dewax solution, and rehydrated in Bond Wash solution (Leica Microsystems). Antigen retrieval was carried out at pH 8 with Epitope Retrieval 2 solution (Leica Microsystems) for 20 minutes at 100°C. Slides were then incubated for 15 minutes at room temperature with the respective primary antibodies at the following dilutions: CD4 (CD4-368-L), 1:100 (4812; Novocastra); CD8 (CB144B), 1:100 (M7103; Dako); CD20 (MJ1), 1:100 (1 mL NCL-CD20-MJ1; Novocastra); CD1a (MTB1), 1:50 (CD1A-235-L-CE; Novocastra); and Granzyme B (GrB-7), 1:50 (M7235; Dako). Antibody detection was carried out with the Bond Polymer Refine Red Detection system as per the manufacturer’s instructions (DS9390; Leica Microsystems). Slides were then counterstained with hematoxylin. The PRE, POST, and PROGRESSION tumors were stained for CD4 and CD8. Only PRE and POST lesions were stained for CD1a, CD20, and Granzyme B.

The slides were examined by 2 investigators (J.S. Wilmott and R.A. Scoyer) who had no knowledge of patient outcome. The above markers were scored in 2 locations in each tumor: in the intratumoral region (within the tumor mass, as shown in Supplementary Fig. 1A), and in the peritumoral region (in the stroma immediately surrounding the tumor, as shown in Supplementary Fig. 1B). The percentage of tumor with lymphocytic involvement and the density of the infiltrate were determined. The density was graded using a 3-tiered system (0, absent; 1, 1–10 isolated positive lymphocytes in any ×40 field of view; 2, 11–50 positive lymphocytes in any ×40 field of view; 3, >50 positive lymphocytes in any ×40 field of view; Supplementary Fig. S1C–S1F). For each lymphocyte subset, an immunoreactivity score (IRS) was derived by multiplying the percentage of tumor involvement by the density score. Microphotographs were taken of areas of interest with an ACIS III microscope (Dako).
### Table 1. Patient details

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Abbreviations: CR, complete response; GSK, GSK2118436; PD, progressive disease; PR, partial response; Roche, PLX4720; SD, stable disease; and UK, unknown.

<sup>a</sup>Patient 1 had low-volume metastatic melanoma and was included in the phase I trial prior to the need for "measurable" disease. Of the 3 metastatic sites, 2 were resected during treatment as part of this TEAM study, and the remaining lesion was a deep muscular deposit seen on PET only. bd, twice a day; tds, 3 times a day.
Statistical analysis
Statistical analyses were conducted with “PASW Statistics 18” SPSS, IBM. Wilcoxon matched-pairs method was used to test for association of immune marker IRS at different treatment stages. Correlations of immune markers with clinical features were conducted using Spearman rho test. Cox regression analysis was used to determine the predictive factors for patient response and outcome.

Results
Patients
There were 37 tumor specimens available for analysis. Twelve patients had paired PRE and POST tumor biopsies, of which 7 also had PROGRESSION tumor biopsies. Three patients had paired PRE and PROGRESSION biopsies only. Of the 15 patients, 2 were treated with vemurafenib and 13 with GSK2118436. Eleven patients had a V600E BRAF mutation and 4 patients had a V600K BRAF mutation. There were 5 women and 10 men with a median age of 39 years (23–71 years). A partial response was achieved in 10 patients, 4 patients had stable disease, and 1 patient had progressive disease (as best investigator-determined response in target lesions based on RECIST criteria as determined by serial CT scans). At the time of last follow-up, 9 patients had died of melanoma and 6 patients were still alive. The median follow-up period was 11 months (range, 2–20 months), with a median time to progression of 5 months (1–13 months).

Figure 1. Immunohistochemical staining for CD8⁺ and CD4⁺ lymphocytes in biopsies taken from patient 4 at different treatment stages (×20 magnification). A, PRE biopsy showing low density of CD8⁺ lymphocytes. B, PRE biopsy negative for CD4⁺ lymphocytes. C, POST biopsy showing high density of CD8⁺ infiltration. D, POST biopsy showing a high density of CD4⁺ lymphocytes. E, PROGRESSION biopsy showing low levels of CD8⁺ infiltration. F, PROGRESSION biopsy showing low levels of CD4⁺ infiltration.
Immune cell expression in melanomas PRE and POST BRAF inhibitor treatment

CD8\(^{+}\) T lymphocytes were present in both the intratumoral and peritumoral regions of the biopsies. Significant increases in intratumoral and peritumoral CD8\(^{+}\) lymphocytes were observed from PRE to POST biopsies (Z = 2.433, ρ = 0.015 and Z = 2.134, ρ = 0.033, respectively; Figs. 1A, 1C, and 2). In contrast, peritumoral and intratumoral CD8\(^{+}\) cells decreased from POST to PROGRESSION biopsies (Z = 2.366, ρ = 0.018 and Z = 1.992, ρ = 0.046, respectively; Figs. 1C, 1E, and 2).

CD4\(^{+}\) T lymphocytes were present in both the intratumoral and peritumoral regions. Intratumoral CD4\(^{+}\) T lymphocytes significantly increased from PRE to POST biopsies (Z = 2.433, ρ = 0.015; Figs. 1B, 1D, and 2). No significant change was observed between intratumoral POST and PROGRESSION biopsies or in peritumoral CD4\(^{+}\) cells from PRE to POST or POST to PROGRESSION.

In POST biopsies, CD4\(^{+}\) and CD8\(^{+}\) lymphocyte IRS scores were significantly positively correlated (r = 0.606, ρ = 0.037). Patients with an elevated CD4\(^{+}\) lymphocytic infiltrate in their POST biopsy also had elevated CD8\(^{+}\) lymphocytes although the number of CD8\(^{+}\) lymphocytes was much higher than that of CD4\(^{+}\) lymphocytes (Fig. 3A). The number of CD4\(^{+}\) and CD8\(^{+}\) lymphocytes did not significantly differ in PRE or PROGRESSION biopsies.

Granzyme B was expressed in a granular pattern in the cytoplasm of lymphocytes. No significant changes in Granzyme B–expressing lymphocytes were observed between PRE and POST biopsies. The numbers of intratumoral CD8

Figure 2. Changes in the IRS scores for immune cell markers between different treatment stages. A, change in IRS of immune cell markers between PRE and POST biopsies (error bars 95% confidence interval [CI]). B–E, line graphs of individual patient IRS scores throughout treatment stages, intratumoral CD8\(^{+}\) (B), peritumoral CD8\(^{+}\) (C), intratumoral CD4\(^{+}\) (D), and peritumoral CD4\(^{+}\) (E). PROG, PROGRESSION.
and Granzyme B–expressing lymphocytes in PRE biopsies were positively correlated ($r = 0.769, \rho = 0.003$). Similarly, the numbers of CD8 and Granzyme B–expressing lymphocytes positively correlated in POST biopsies ($r = 0.690, \rho = 0.013$; Fig. 3B).

CD20$^+$ cells were located in the intratumoral and peritumoral regions. No significant change in CD20$^+$ lymphocytes was observed between PRE and POST biopsies. The scale of increase in CD20$^+$ B lymphocyte infiltration was significantly less than that of CD8$^+$ T cells in the intratumoral region of POST biopsies ($Z = 3.061, \rho = 0.002$; Fig. 3C). No significant difference was observed between CD20$^+$ and CD8$^+$ IRS in PRE biopsies.

CD1a$^+$ dendritic cells were identified in the epidermis of normal skin present within some of the biopsies (i.e., positive internal control). Most tumors were negative for CD1a$^+$ cells; only very occasional CD1a$^+$ dendritic cells were present in the POST biopsies of 2 patients.

**Association of TILs and tumor response**

The change in CD8$^+$ T-cell infiltration from PRE to POST biopsies in both intratumoral and peritumoral areas correlated with a decrease in the POST biopsy caliper measurements of tumor size ($r = -0.793, \rho = 0.011$ and $r = -0.834, \rho = 0.005$, respectively, Fig. 4A). Similarly, an inverse correlation was observed between the change from PRE to POST biopsy peritumoral CD8$^+$ lymphocytic infiltration and the reduction in metabolic activity of target lesions ($r = -1.00$, although $n = 6$). The intratumoral Granzyme B$^+$ T-lymphocyte infiltration of POST biopsies was inversely correlated with the change in caliper size in the POST biopsy and the overall change in metabolic activity of all metastases from baseline to day 15 ($r = -0.858, \rho = 0.003$ and $r = -0.841, \rho = 0.036$, respectively). POST biopsy intratumoral CD4$^+$ lymphocyte infiltration inversely correlated with the change in the overall metabolic activity of all metastases from baseline to day 15 ($r = -0.841, \rho = 0.036$). Intratumoral CD8$^+$ and CD4$^+$ lymphocytic infiltration in POST biopsies positively correlated with the percentage of tumor that was necrotic ($r = 0.761, \rho = 0.004$ and $r = 0.618, \rho = 0.32$, respectively; Fig. 4B and C).

**Association of TILs and clinical response**

There was no association between the changes in lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the association of TILs and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival. Cox regression analysis of the lymphocytic infiltration and CT tumor response, time to progression, or overall survival.

**Discussion**

Clinical trials with the potent immunostimulant ipilimumab have reported 2-year survival of more than 30% in patients with AJCC stage IV metastatic melanoma, and disease control tended to correlate with clinical evidence of immune stimulation (17, 18). Given the promising early results of clinical trials of selective BRAF inhibitors (6–9), understanding the immune response to melanoma following selective BRAF inhibitor treatment is critical to the development of therapies based on combination of BRAF inhibitors with immunotherapies such as ipilimumab (17, 18). In this study, we found an increase in CD8$^+$ and CD4$^+$ TILs in response to the BRAF inhibitor treatment early after commencement. The CD8$^+$ cytotoxic T cells increased significantly more than CD4$^+$ Th cells. CD20$^+$ B cells did not change following BRAF inhibitor treatment. Importantly, the increase in CD8$^+$ lymphocytes correlated with a decrease in the size and metabolic activity of tumors. The early increases in the density of lymphocytes that occurred initially following BRAF inhibitor treatment were reduced in the biopsies taken following disease progression.

These findings corroborate the results of in vitro studies conducted on cell lines that suggest BRAF inhibitors do not compromise immune cell function and may increase immune cell recognition of melanoma cells (12, 13). The current study suggests that the treatment of patients with a BRAF inhibitor may increase both Th-cell and cytotoxic T-cell responses against the tumor and that this may contribute to their therapeutic effects. Previous studies have shown that increased expression of Granzyme B–expressing CD8$^+$ (active) and CD4$^+$ cells in stage II melanoma biopsies correlated with a favorable outcome (19). The results in the current study showing that an increase in the number of CD8$^+$ and Granzyme B$^+$ T cells correlated with a decrease in posttreatment biopsied lesion diameter are consistent with these findings. No significant association was observed between immune cell markers and patient outcome, although a larger cohort of patients may be needed to establish such a correlation.

The infiltrates of CD8$^+$ and CD4$^+$ T cells were significantly less in tumors excised following disease progression compared with those taken posttreatment, and were similar to the levels in pretreatment biopsies. A similar association was observed for peritumoral infiltrates of CD8$^+$ lymphocytes. These results, together with the inverse correlation between POST biopsy CD8$^+$ lymphocyte infiltration and the change in caliper-measured size of the POST tumors, suggests that the intratumoral infiltration by CD8$^+$ and CD4$^+$ T cells was dependent on tumor sensitivity to the BRAF inhibitor. This would be consistent with inhibition of lymphocyte infiltration due to the release of inhibitory cytokines or other factors from the tumor as it became less responsive to the BRAF inhibitor. Nevertheless, the observation of a positive correlation between CD8$^+$ expression and necrosis suggests that the infiltration by lymphocytes may in part be mediated by melanoma cell death induced by the BRAF inhibitors.

The amount of tumor tissue available was not sufficient for a more detailed analysis of the lymphocyte subsets. Future studies assessing suppressor T cells such as FoxP3, as well as expression of checkpoint inhibitors such as CTLA4, Programmed Death 1 (PD1), and T-cell immunoglobulin mucin 3 (TIM3) and their respective ligands on the melanoma cells, may provide helpful information.
These studies, together with a measure of immunosuppressive cytokines and antiapoptotic proteins in the melanoma cells, as well as a more comprehensive analysis of dendrite cell subsets, may provide a more precise biologic characterization of the immune response to treatment with the BRAF inhibitors and allow more accurate correlation with clinical outcomes (20).

In conclusion, we report that there was a marked early increase in TILs in patients with metastatic melanoma following BRAF inhibitor treatment, consisting mainly of...
CD8⁺ cytotoxic T cells. The mechanisms involved in this increase in TILs are yet to be defined, but prior studies would support the view that this may involve suppression of the release of immunosuppressive factors from the melanoma and increased melanoma antigen expression leading to more effective T-cell recognition. These results provide strong support for future trials combining selective BRAF inhibitors with immunotherapeutic agents such as ipilimumab. Optimally, this may result in increased durability of the high response rates seen with the BRAF inhibitors due to the concomitant immunotherapy with ipilimumab.

Disclosure of Potential Conflicts of Interest
R.F. Kefford has honoraria from and is a consultant for Roche and GlaxoSmithKline. G.V. Long is a consultant for Roche, Bristol-Myers.
Squibb, and GlaxoSmithKline and has honoraria from Roche and travel support from GlaxoSmithKline. R.A. Scolyer is a consultant for Roche and GlaxoSmithKline and has honoraria from Abbott Molecular. No potential conflicts of interest were disclosed by the other authors.

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