Companion Diagnostics and Cancer Biomarkers

Clinical Utility of a Blood-Based BRAFV600E Mutation Assay in Melanoma

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Abstract

BRAF inhibitors (BRAFi) have led to clinical benefit in patients with melanoma. The development of a blood-based assay to detect and quantify BRAF levels in these patients has diagnostic, prognostic, and predictive capabilities that could guide treatment decisions. Blood BRAFV600E detection and quantification were performed on samples from 128 patients with stage II (19), III (67), and IV (42) melanoma. Tissue BRAF analysis was performed in all patients with stage IV disease and in selected patients with stage II and III disease. Clinical outcomes were correlated to initial BRAF levels as well as BRAF level dynamics. Serial analysis was performed on 17 stage IV melanoma patients treated with BRAFi and compared with tumor measurements by RECIST. The assay was highly sensitive (96%) and specific (95%) in the stage IV setting, using a blood level of 4.8 pg as “positive.” BRAF levels typically decreased following BRAFi. A subset of these patients (5) had an increase in BRAFV600E values 42 to 112 days before clinical or radiographic disease progression (PD). From 86 patients with resected, stage II or III melanoma, 39 had evidence of disease relapse (45.3%). Furthermore, BRAF mutation in the blood after surgical resection in these patients was not associated with a difference in relapse risk, although tissue BRAF status was only available for a subset of patients. In summary, we have developed a highly sensitive and specific, blood-based assay to detect BRAFV600E mutation in patients with melanoma. Mol Cancer Ther; 13(12); 3210–8. ©2014 AACR.

Introduction

Metastatic melanoma is currently the fifth and seventh most common cancer in American men and women, respectively, and remains one of the few cancers with a rising incidence (1). In the United States, more than 9,000 people died from this disease in 2013 (1). Recent treatment advances have led to the FDA approval of two BRAF inhibitors, vemurafenib (Zelboraf) and dabrafenib (Tafinlar), a MEK inhibitor, trametinib (Mekinist), and the immunotherapy ipilimumab (Yervoy) for the treatment of patients with advanced melanoma (2–6). Unfortunately, resistance to BRAF and MEK inhibitor therapy is common, response to ipilimumab uncommon, and durable response to any therapy infrequent; as such, the overwhelming majority of these patients eventually will die of their disease (7, 8). Most patients with BRAF-mutant disease will be candidates for multiple lines of therapy, but conventional radiographic monitoring to track response and progression fails to identify patients at a point when they can receive benefit from follow-on therapy. There is a critical need to develop highly sensitive blood-based biomarkers that could enable better treatment selection and improved monitoring of patients with advanced and high-risk melanoma.

Current, standard BRAF testing methods are tissue based and provide only qualitative data, i.e., positive or negative (9–14). The major limitations to these approaches are lack of sensitivity and the need to acquire tissue (either via location of an archived tumor block or fresh biopsy). Most tissue-based assays have the ability to identify one mutant allele in 10 or 20 wild-type alleles and thus require tumor specimens that contain approximately 40% to 50% tumor cellularity to account for heterozygosity and stromal and lymphoid elements typically present in melanoma metastases (9–15). Although most metastatic tumor biopsies have little trouble meeting this benchmark, analysis of primary melanomas and microscopically involved sentinel nodes are less reliable due to tumor heterogeneity (primary tumors) and/or relative infrequency of tumor...
cells (sentinel lymph nodes; refs. 16, 17). Furthermore, the identification of an appropriate block or the coordination of biopsy and subsequent analysis delays the start of systemic therapy. In these circumstances, a highly sensitive blood-based assay would provide a superior diagnostic tool.

A blood-based assay also would provide serial data about the state of the disease. For example, patients with resected melanoma have a risk of recurrence and death that ranges from 7% to 80%. Although clinical and pathologic staging can narrow the range, it is still broad for each stage of cancer and serial blood testing and imaging is of little value in improving prognostic accuracy (18). An assay that rises in the setting of disease would likely enhance the predictive value of imaging and allow for timely diagnosis and treatment of recurrent melanoma. During the treatment of the metastatic disease, blood tests that can serve as a surrogate marker of disease status and substitute for more expensive and difficult radiographic imaging would offer a cost effective option to imaging and allow earlier transition to next-line therapy for patients with emerging resistant disease.

We previously described the development of a highly sensitive and inexpensive, blood-based BRAF assay that took advantage of a unique restriction enzyme site in wild-type BRAF at the V600 position (19). Our current assay continues to target this restriction digest site and also adds a real-time PCR (RT-PCR) step that allows for precise quantification of BRAF levels. In this report, we describe this enhanced assay and present testing results from patients with stage II, III, and IV melanoma.

### Materials and Methods

#### Cell lines, tissue acquisition, and oligonucleotides

The melanoma cell line A375, kidney cancer cell line 786-0, colon adenocarcinoma HT29, and prostate carcinoma DU145 were purchased in 2013 from the ATCC. All four cell lines were authenticated by isoenzymology and the Cytochrome C subunit I (COI) PCR assay was performed for confirmation of species. In addition, the cell lines had their identity confirmed by short tandem repeat (STR) analyses. Oligonucleotides were custom synthesized from Invitrogen and Sigma.

#### The protocol in brief

The protocol is a quantitative modification of a previous assay. As illustrated in Fig. 1A, an initial RT-PCR is followed by digestion with TspR1 (restriction site, NNCASTGNN; New England Biolabs) at 65°C for 16 hours. Only wild-type BRAF and not V600E-mutant BRAF PCR product was digested by this enzyme. This digestion was added to reduce the amount of contaminating normal BRAF from surrounding and infiltrating normal tissue in the blood samples. A 1:100 dilution of the TspR1 digested material was then PCR amplified a second time using nested oligonucleotides 5′(ACGCCAAGTCAATCATACTCCACAGAG)3′ and 5′(CCCCACACCTTACACTGAGACAGAC)3′ producing a product of 331 bp, which was enriched in PCR products containing the position 600 mutation. The conditions of the PCR were the same as the first PCR except the amplification was 45 cycles for PBLs instead of 40 cycles. After a second cleanup using a NucleoSpin Extract Column, the DNA (1:1,000 dilution) was digested again with TspR1 and then subjected to a BRAFV600E RT-PCR as described (21). The annealing and extension temperature was adjusted to 64°C resulting in a more favorable amplification of the mutant as compared to the wild-type templates (Fig. 1B) than was reported (21). To further favor the mutant over the wild-type product, a 32-fold excess of the reverse (common sequence in mutant and wild-type) to forward (exact match for mutant and one-base mismatch for wild-type sequences) primers were used in the RT-PCR assay. Therefore, after two rounds of TspR1 digestion, it is highly unlikely that any remaining wild-type product would have a significant impact on the assay. Purified BRAFV600E first-round PCR product with a known concentration was also run through the assay and was used to create a standard curve. Using the standard curve, the amount of end product was determined.

#### Peripheral blood isolation

PBLs were obtained from 128 patients with advanced or cutaneous melanoma as part of IRB-approved tissue-collection protocols.
banking protocols (DFHCC 02–017 and 11–181). PBLs were isolated by Ficoll density centrifugation (22). Of these 128 patients, 42 had stage IV disease and had blood collected specifically for this analysis between 2009 and 2012. PBL from the 19 patients with stage II melanoma and 67 patients with stage III disease were collected and isolated approximately 4 to 8 weeks following completion of surgical management as part of the Harvard Skin SPORE between 2002 and 2006. These samples were stored in freezing medium (95% fetal calf serum with 5% DMSO) at −80°C (stage IV samples) or in liquid nitrogen (stage II and III samples). Furthermore, blood was drawn pre- and post-resection from 8 stage III patients previously determined to be BRAFV600E by tissue analysis. Only one sample per patient was available for most patients involved in this study and in all of the patients with stage II or III disease (except for the aforementioned 8 patients with stage III disease with pre- and postoperative samples).

Serial blood samples were collected and assayed from 12 patients receiving the BRAF inhibitor vemurafenib and 6 patients receiving the combination of dabrafenib and trametinib. Samples were collected until RECIST-determined disease progression was documented in 17 of these 18 patients. In a subset of patients, different tubes were used after initiation of BRAF-directed therapy. Specifically, CPT tubes were used and the cellular component was removed and analyzed. Analyses comparing the two techniques (Ficoll isolation and CPT tube isolation) revealed a 7.5-fold greater BRAF level when Ficoll was used compared with CPT tube isolation (Supplementary Fig. S1). As a result, only samples isolated using Ficoll isolation were analyzed in this study.

Tissue-based BRAF analysis
Patients with stage IV melanoma had BRAF mutational analysis on tumor tissue as part of standard of care either via the Cobas assay (Clariant Labs) or via SNaPshot (Massachusetts General Hospital Cancer Center Translational Research Laboratory; refs. 23, 10).

Figure 1. A, schematic of the BRAFV600 assay; B, standard curve of the BRAF assay. The equation representative of the best fit line for the BRAFV600E (lower equation) and wild-type BRAF (upper equation) are shown. C, BRAF expression level in individual cell lines, including a homozygous BRAFV600E-mutant line (A375), two BRAF wild-type lines (786-0, Du145), and a heterozygous line (HT29). D, ROC curve for stage IV BRAFV600E patients. With AUC of 0.9929, the assay has excellent classification ability for stage IV melanoma.
Statistical analysis

Summaries of BRAF expression levels over time are presented using descriptive methods. Comparisons of BRAF expression according to mutational status or stage of disease were based on linear regression models of natural log(BRAF) with mutational status or stage as the single predictor. Bonferroni corrections were used for pairwise comparisons to adjust for multiplicity. The distributions of relapse-free survival (RFS) and overall survival (OS) are described using the method of Kaplan–Meier. Five-year estimates of RFS and OS are presented with 95% confidence intervals calculated using log-log method. Statistical significance is defined as $P < 0.05$; there are no adjustments for multiple comparisons.

Results

Generation of standard curves

Standard curves were generated using known amounts of purified primary PCR products of wild-type and V600E BRAF as templates. The assay can reliably detect as low as 1 pg of BRAFV600E and exhibits a nearly 1,000-fold difference in sensitivity for the V600E as compared with wild-type BRAF PCR product (Fig. 1B).

Sensitivity and specificity of assay

To examine the specificity of the assay, the protocol was used in four cell lines: A375, a melanoma line with a homozygous BRAFV600E mutation, HT29, an adenocarcinoma that is heterozygous for the BRAFV600E and 786-0 and Du145, renal cell carcinoma (RCC) and prostate cell lines, both wild type for BRAF. As shown in Fig. 1C, using equal amounts of input RNA (3 μg), the A375 and HT29 cells expressed nearly 10,000-fold greater BRAFV600E than either wild-type cell line. Using a cutoff value of 4.8 pg in blood samples, the sensitivity of the assay for stage IV patients is 96% and the specificity 95%. The area under the receiver operator curve (ROC) was 0.9929, demonstrating an excellent ability to discriminate patients with and without BRAF-mutant melanoma (Fig. 1D).

Samples obtained from 128 (42 stage IV, 67 stage III, and 19 stage II) patients with melanoma were analyzed. A comparison of BRAF levels from 42 stage IV melanoma patients whose tumor biopsies had been previously determined to contain a V600E-mutant (27 patients) or wild-type BRAF (15 patients), 4 RCC patients (known to be BRAF WT), and two normal controls was performed (Fig. 2A). Mean BRAFV600E levels were 50.3, 1.7, and 1.2 pg for patients with mutant disease, wild-type melanoma, or RCC/nonmelanoma, respectively ($P < 0.0001$).

In the 86 patients with stage II and III melanoma, the median BRAF value from the postresection blood draws was 0.48 pg, regardless of the BRAF status by tissue analysis. A comparison of BRAF values in patients across stage II, III, and IV show that patients with stage IV melanoma had significantly higher blood BRAFV600E values (Fig. 2B).

Thirty-seven of the 67 stage III patients had tumor blocks suitable for tissue BRAF mutation analysis. In these patients, tissue-based analysis detected a BRAFV600E mutation in 17 patients. Using a cutoff value of 4.8 pg (determined from the stage IV ROC curve) in blood samples, 5 of the 17 patients with tissue BRAF detection had an elevated blood value. Furthermore, the 10 highest postoperative BRAFV600E levels in these patients were all patients with known BRAF mutations based on tissue analysis and none of the 20 patients without BRAFV600E

![Figure 2](https://mct.aacrjournals.org/content/13/12/3213/F2.large.jpg)

Figure 2. Distributions of BRAF levels by mutation status (A) and disease stage (B). BRAF levels were transformed using natural logarithms (base e). A, BRAFV600E level is higher in patients with tissue-detected BRAF mutation than in patients with stage IV BRAF WT melanoma ($P < 0.0001$), and patients without melanoma (normal; $P < 0.0001$); B, BRAFV600E level is higher among patients with stage IV, BRAF-mutant melanoma than in patients with stage II and III melanoma with a "positive" BRAF level ($P < 0.0001$). Statistical comparisons based on linear models with disease stage or mutation status as single predictor. Bonferroni corrections were used to adjust for multiple comparisons.
detection in tissue had an elevated V600E value (Fig. 3A). Of note, the mean BRAF value in the 17 patients with known BRAF mutation in tissue was 21 pg compared with 0.26 pg in the 20 patients without identified mutation in tissue, although this difference did not meet statistical significance ($P < 0.128$). To potentially explain the high number of postresection blood samples that had negative BRAF V600E levels despite being tissue positive, pre- versus postresection samples were compared. All samples were previously determined to be BRAF V600E by tissue analysis. As shown in Fig. 3B, all 8 patients showed a marked decrease in BRAF V600E levels postsurgery. Furthermore, 5 patients had postoperative levels below the level of detection (4.8 pg) for this assay. The reduced tissue burden as a result of surgical resection provides a plausible explanation for the approximate 70% discordance between tissue and postsurgical blood analysis by this assay. In addition, comparisons in all 86 stage II and III patients of blood BRAF values postsurgery were made according to stage (stage II vs. stage III), substage (stages II and III A, B, and C), and intermediate versus high risk, defined as risk of death < 50% (AJCC stage IIA, IIB, or IIIA) or ≥ 50% (stage IIC, IIIB, IIIC), and showed no significant differences.

**Predictive value of V600E BRAF blood in stage II and III melanoma**

In the 86 patients with resected, stage II or III melanoma, 39 had evidence of disease relapse (45.3%). In all these patients, detectable oncogenic BRAF mutation in the blood was not associated with a difference in the risk of relapse (5-year RFS; 52% vs. 57%, log-rank $P = 0.98$) or death (5-year OS; 73% vs. 75%, log-rank $P = 0.88$). Analysis of BRAF levels quartiles similarly showed no evidence of OS difference (Fig. 3C). These findings show that, although not predictive of outcome at this time, this assay can detect the BRAF V600E mutation in resected stage II and III patients, regardless of the tumor BRAF status.

**Analysis of serial blood samples from patients receiving BRAF targeted therapy**

Blood BRAF V600E levels in 18 patients with BRAF V600E melanoma treated with either vemurafenib (12 patients) or the combination of dabrafenib and trametinib (6 patients) dramatically reduce following the commencement of therapy (Fig. 4), and this reduction is similar in patients treated with single-agent BRAF or combination BRAF–MEK inhibitor therapy. Supplementary Fig. S2A–S2C shows the serial values of the BRAF V600E level in the
Blood of 11 patients treated with single-agent vemurafenib and all 6 patients treated with dabrafenib and trametinib in whom tumor measurements from serial CAT scans acquired by patients until disease progression were also plotted alongside the blood BRAFV600E data. In the majority (15 of 17) of patients, a reduction in blood BRAFV600E level correlated with disease response on imaging. After the decrease in BRAFV600E, 5 of the 17 patients showed an increase in BRAFV600E in the blood 42 to 112 days before having treatment stopped because of disease progression (Supplementary Fig. S2C). Of note, in all 17 patients, treatment was halted because of the occurrence of new lesions or nontarget lesion progression and not due to target lesions progression.

Discussion

Blood-based detection of oncogenic mutations has become increasingly widespread. Alternative methods make use of RT-PCR, mass spectrometry, allelic-specific PCR, PCR using locked oligonucleotides to suppress wild-type sequences, direct sequencing of RNA or DNA to preferentially distinguish the mutant V600E from wild-type BRAF, as well as a combination of emulsion-based digital PCR and flow cytometry (so-called Beads, Emulsion, Amplification, and Magnetics or BEAMing; refs. 9, 15, 24–31). Our assay is unique due to our approach that leads to its high sensitivity and specificity. This is achieved by both the use of RNA from PBLs isolated from Ficoll, as it is postulated these cells contain either circulating tumor cells (CTC) or other encapsulated, circulating RNA-containing factors released by tumors, and the reduction of background from wild-type BRAF with the use of TspR1, a restriction enzyme that preferentially digests only the wild-type sequence from the first PCR product (19, 21). As a result, we have a highly sensitive assay (96% in patients with active, metastatic disease) that has exquisite specificity (95% in these same patients) and is able to provide quantitative information due to the use of V600E-specific RT-PCR.

An initial application of this assay would be to diagnose BRAF-mutant disease. Current tissue-based BRAF mutational methods can be challenging in archived primary melanomas (tumor heterogeneity) and microscopic nodal metastases (lack of sensitivity), and patients with newly metastatic melanoma often have rapidly progressive disease that requires urgent identification of mutational analysis. Analyzing blood for the BRAF mutation would prove to be a more efficient and possibly more reliable method of determining a patient’s BRAF status. The sensitivity and specificity seen with our assay are encouraging, although are limited by a relatively small sample size. Confirmation of this degree of sensitivity and specificity is required before broad-scale, blood-based analysis as the sole determinant of whether a melanoma harbors a BRAF-mutation can be adopted.

A second use of this assay would be in the stage II and III setting, as the optimal follow-up of patients who are rendered disease-free with surgery for their melanoma is unknown (32). The development and validation of a blood-based prognostic biomarker would offer the potential to improve the NCCN guidelines and direct radiographic imaging. Our data show that while blood BRAFV600E levels reduce following surgery, postoperative blood-based analysis may be useful in diagnosing BRAFV600E status. The major weakness with these data, however, is that in the majority of cases tissue analysis was not performed because of either a lack of access to tumor blocks or insufficient disease in the blocks. Still, we demonstrate proof-of-concept that blood BRAFV600E levels can be detected in the blood of patients with resected melanoma. A second weakness is that we only

Figure 4. Blood BRAFV600E levels in 18 patients with BRAFV600E melanoma treated with either vemurafenib (12 patients) or the combination of dabrafenib and trametinib (6 patients) dramatically reduced following the commencement of therapy. A, the data show the response of individual patients to the drug regimen. B, mean BRAFV600E levels in response to either vemurafenib or the dabrafenib and trametinib combination. The values in B are relative to pretreatment levels (set as 1.0).
have one postoperative blood value in the majority of these patients. It is conceivable that the BRAF level would change over time and that this change in level over time might be more predictive of outcome than a one-time value. Investigation into this specific application of this assay is under way (NCT01840527).

A third potential use of our assay would be to help guide the use of adjuvant therapy in patients with BRAF-mutant melanoma. There are two open phase III trials (NCT01667419, NCT01682083) testing the effectiveness of BRAF-directed therapy in patients with resected, high-risk disease; it is conceivable that adjuvant BRAF-directed therapy will become standard of care. If so, it is clear that the presence of a BRAF mutation in tissue will be requisite to qualify for this type of therapy. However, assessment of BRAF mutations in primary melanoma samples may be complicated by substantial tumor heterogeneity among cells in the primary tumor and between primary and metastatic tumors (16, 17). This may lead to both false negatives and false positives, as it may not be clear which clones will ultimately establish metastasis. Our analysis of 37 patients with either stage II or III disease who had both tissue and blood analysis performed showed congruous findings (i.e., both positive or both negative) in 25 (67%). Importantly, all 20 wild-type patients by tissue analysis were also wild-type by blood analysis. Of the 17 patients with BRAFV600E by tissue analysis, only 5 were also V600E by blood. A plausible explanation for the 12 patients with a BRAF mutation identified in tissue without detectable mutation in the blood may have had such low tumor volume following surgery that the circulating BRAF levels were below the limits of detection; thus a scenario where both the tissue and blood analysis may be true yet discordant. An alternative explanation would be that either the tissue analysis was falsely positive or the blood assay falsely negative. Prospective collection and analysis is under way in both the stage II and III setting to better define the utility of this assay in this setting (NCT01840527).

Finally, an assay that has the potential to identify tumor resistance to BRAF-directed therapy at an earlier time point is desperately needed. BRAFi resistance typically develops within 6 to 8 months following initial tumor regression, but with a range of 2 months to 2 years (2, 4, 33, 34). Importantly, each described mechanism involves the retention of the initiating BRAF mutation (35–43). As the mechanisms of resistance are now being elucidated, we feel that diagnostic assays, which may identify emerging resistance at an earlier time point than standard clinical or radiographic assessments, will enable more prompt switching to another therapy. This is particularly important due to the fact that a number of patients treated with BRAF inhibitors progress quite quickly following initial disease regression (44, 45). Although there are no current data that specifically support such a strategy, it is thought that more advanced notice of disease progression, when disease growth is more modest, would allow for a more timely change in treatment and improved benefit of next-line therapy. To date, only a small number of patients have been followed serially (17 presented here) with our assay in the context of BRAF-directed therapy. Still, this is the largest number presented to date followed with serial testing with a quantitative BRAF assay. Our findings—that BRAF level reduces with the initiation of BRAF inhibitor therapy and typically increases at the time of or in advance of radiographically defined disease progression—are compelling. Although these findings require confirmation, they also serve as a proof of concept that this type of assay may have value in this patient population and treatment setting.

There are a number of limitations of this study that serve to temper enthusiasm for the widespread adoption of this assay. First, our assay only measures BRAFV600E levels and is likely not useful for patients with BRAFV600K, or another non-V600E, V600 BRAF mutation. Second, all the samples used in the stage II and III analysis were obtained between 2001 and 2006, whereas all the samples used for the stage IV analysis were collected prospectively from 2010 to 2012. It is very possible that long-term storage (in this case stored frozen in liquid nitrogen) might affect the levels of BRAF expression. Third, it is difficult to interpret the stage II and III data in the absence of corresponding tissue BRAF mutational analysis. Given the change of accurate testing of primary tumors and microscopically involved lymph nodes, this is a problem that may never be completely solvable, although prospective collection will likely lead to a higher percentage of patients with available tissue amenable to tissue-based analysis. Fourth, the fact that we were analyzing samples from a retrospective cohort meant that we only had one time point available for analysis in the majority of these stage II and III patients. Depending on just one time point in the clinical care continuum of these patients reduces the chances of showing the prognostic value of this assay. Fifth, the serial analyses of the BRAF-mutant melanoma patients treated with BRAF-directed therapy involves a small number of patients with variable time points obtained. In particular, the on-treatment blood draws were initially going to be obtained at 2 weeks, 4 weeks, and then every 4 weeks; but soon after we changed this to every 4 weeks. Still, the inclusion of all patients allows for a more comprehensive look at this assay.

In conclusion, we have reported the clinical utility of the first blood-based, BRAF detection and quantification assay in a number of clinical settings. Although our findings require confirmation before more extended adoption of this or similar clinical assays, the data provide proof-of-concept that circulating blood-BRAF can be collected, quantified, flowed, and utilized in patients with stage II, III, and IV melanoma.

Disclosure of Potential Conflicts of Interest

D.F. McDermott is a consultant/advisory board member for Genentech. J.A. Wargo has speakers’ bureau honoraria from Dava Oncology and is a consultant/advisory board member for Genentech and GlaxoSmithKline. F.S. Hodi reports receiving Genentech trial support to the institution and is a non-paid consultant for Genentech. M.B. Atkins is a consultant/advisory board member for Genentech, GlaxoSmithKline, and Bristol-Myers Squibb. K.T. Flaherty is a consultant/advisory board member of Bristol-Myers Squibb.
member for GlaxoSmithKline, Novartis, and Roche/Genentech. No potential conflicts of interest were disclosed by the other authors.

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