Utility of Circulating *B-RAF* DNA Mutation in Serum for Monitoring Melanoma Patients Receiving Biochemotherapy

Masaru Shinozaki,[1] Steven J. O'Day,[4] Minoru Kitago,[1] Farin Amersi,[1,2] Christine Kuo,[1] Joseph Kim,[1,2] He-Jing Wang,[3] and Dave S.B. Hoon[1]

Abstract

Purpose: Somatic *B-RAF* gene mutation has been identified in many malignancies and detected at a high frequency in cutaneous malignant melanoma. However, the significance of the *B-RAF* mutation (*B-RAF*mt) in terms of its prognostic and predictive capabilities for treatment response or disease outcome is not known. We hypothesized that circulating serum *B-RAF*mt (*B-RAF*smt) at V600E, detected in serum, predicts response in melanoma patients receiving concurrent biochemotherapy.

Experimental Design: A real-time clamp quantitative reverse transcription-PCR assay was designed to assess *B-RAF*smt by peptide nucleic acid clamping and a locked nucleic acid hybrid probe. Normal (n = 18) and American Joint Committee on Cancer stage I to IV melanoma patients (n = 103) were evaluated. These included stage IV patients (n = 48) with blood drawn before and after biochemotherapy. Patients were classified as biochemotherapy responders or nonresponders. Responders (n = 24) had a complete or partial response following biochemotherapy; nonresponders (n = 24) developed progressive disease.

Results: Of the 103 melanoma patients, 38 (37%) had *B-RAF*smt DNA, of which 11 of 34 (32%) were stage I or II, and 27 of 69 (39%) were stage III or IV. Of the 48 biochemotherapy patients, 10 of 24 (42%) patients were positive for the *B-RAF*smt in the respective responder and nonresponder groups before treatment. After biochemotherapy, *B-RAF*smt was detected in only 1 of 10 patients (10%) in the responder group and 7 of 10 patients (70%) in the nonresponder group. *B-RAF*smt is associated with significantly worse (P = 0.039) overall survival in patients receiving biochemotherapy.

Conclusion: These studies show the presence and utility of circulating *B-RAF*smt DNA in melanoma patients.

The management of cutaneous melanoma continues to pose a significant challenge. Clinical prognostic factors have not been shown to predict disease recurrence and overall survival in patients with metastatic disease. Adjuvant therapy for melanoma can have major side effects and can be associated with significant morbidity. In addition, it has been difficult to identify which patients will respond to the few treatment options available and to predict disease recurrence and progression.

Over the last decade, advances in melanoma translational research have attempted to identify key components in molecular and genetic alterations that affect the progression of this disease (1). High-throughput genomic approaches have been focused on identifying gene aberrations in the RAS-RAF-mitogen-activated protein/extracellular signal-regulated kinase (MAP/ERK) and (MEK)-ERK-MAP kinase (MAPK) signaling pathways because they have been shown to regulate cellular differentiation, proliferation, and apoptosis (2–4).

B-RAF mutations (*B-RAF*mt) have been reported at a high frequency in melanoma, thyroid, and lung cancer (5–8). *B-RAF* encodes a serine/threonine kinase downstream for RAS in the MAPK pathway that transduces regulatory signals from RAS through MAPK (8–11). *B-RAF*mt have been found at multiple sites, whereby clustering around exons 11 and 15 of the gene in the kinase domain is quite frequent (5, 6, 12). In our recent study evaluating the frequency of *B-RAF*mt in melanoma progression, we found that the V600E (formerly V599E) amino acid missense mutation resulting from a 1796T → A transversion in exon 15 of *B-RAF* was the predominant mutation in the tumors assessed, and the mutation was found in 31% of primary melanoma and 57% of metastatic melanoma tumors (13). Because this mutation has been shown to significantly increase kinase activity and occurs at a significantly higher frequency than other gene mutations found in melanoma, such as *N-RAS*, $p16^{INK4a}$, and *p53* (5, 14, 15), we hypothesized that the presence of circulating DNA with *B-RAF*mt at V600E in the

Authors' Affiliations: [1]Department of Molecular Oncology, Divisions of [2]Surgical Oncology and [3]Biostatistics, John Wayne Cancer Institute at Saint John's Health Center and [4]The Angeles Clinic and Research Institute, Santa Monica, California
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Requests for reprints: Dave S.B. Hoon, Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA, 90404. Phone: 310-449-5267; Fax: 310-449-5282; E-mail: hoon@jwci.org.

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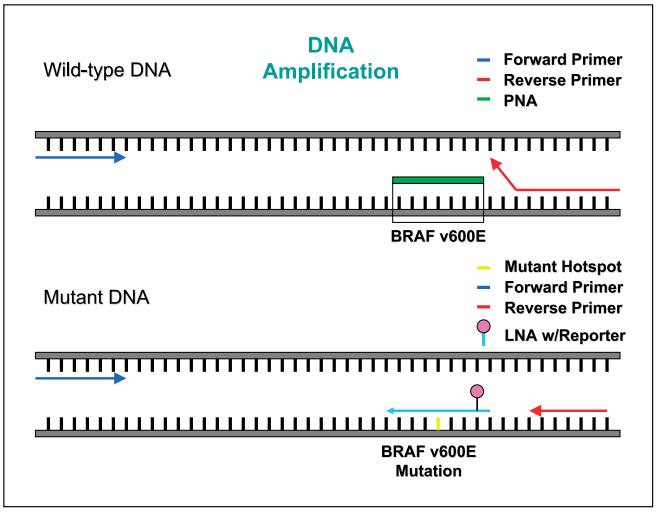

Fig. 1. Schematic of PNA/LNA clamp directed PCR. Top, PNA/wt DNA complex, with no amplification. Bottom, amplification of DNA template containing *B-RAF*mt using the dual-labeled LNA probe that recognizes and hybridizes to V600E.

serum of melanoma patients may be clinically relevant. We have previously shown that circulating DNA in the serum of melanoma patients has clinical utility as a marker for disease progression, identification of occult recurrences, and predicting response to surgical and adjuvant therapy (16–18). Mori et al. showed a correlation between circulating methylated DNA in serum and disease progression and showed an association between circulating methylated DNA and response to biochemotherapy (16, 19).

In this study, we developed a peptide nucleic acid (PNA) clamp– and locked nucleic acid (LNA) probe technique–based quantitative real-time PCR assay to detect serum-circulating *B-RAF*smt DNA of melanoma patients. The detection of single base pair mutations in circulating DNA requires a very sensitive assay because the frequency of circulating *B-RAF*smt DNA will be low. PNAs and LNAs are high-affinity DNA synthetic analogues that hybridize with complementary DNA (20). PNAs have *N*-(2-aminoethyl)-glycine units as backbones. PNA-DNA hybrids are more stable than those for cDNA-DNA and are highly sensitive and specific in distinguishing single base pair mismatches. In addition, as PNA oligomers cannot function as primers in PCR reactions, they are used as blockers to prevent amplification of wild-type DNA templates (21). LNAs also have higher affinity to DNA than cDNA and were incorporated into our assay for their specificity in recognizing single base pair mismatches. LNA-DNA chimeras can be constructed for use as primers or probes. Highly specific detection of *B-RAF*smt was achieved using a specific PNA clamping and LNA hybridizing probe.

The purpose of this study was to determine whether we could identify *B-RAF* V600E mutation on exon 15 as circulating DNA in the serum of melanoma patients and to determine whether quantitative detection of the *B-RAF*mt could have potential clinical applicability in evaluating noninvasive disease progression or quantitative evaluation of therapeutic maneuvers. To date, detection of *B-RAF*mt (V600E) in melanoma tissue has not shown any significant correlation to disease outcome, although *B-RAF*mt in metastatic melanoma can be frequently detected in >55% of patients. In this study, we detected amounts of circulating mutant DNA with high sensitivity and specificity. To further investigate the possible clinical implications of the presence of the *B-RAF*smt DNA, we collected sera from patients before and after treatment with biochemotherapy. We hypothesized that the presence of *B-RAF*smt in posttreatment serum may indicate absence of response to treatment.

Materials and Methods

Patients and cell line. Fifty-five patients with different American Joint Committee on Cancer stages of melanoma were assessed for *B-RAF*smt. In addition, 50 stage IV melanoma patients who received biochemotherapy were also included for the treatment response study. Blood was drawn within 1 week before the start of biochemotherapy and within 4 weeks after the last cycle. The median completed cycles of biochemotherapy were six for the responder group and three for the nonresponder group. The maximum number of cycles received were six cycles, as previously described (22, 23). These patients were further divided into two groups based on their response to biochemotherapy (responders and nonresponders). Patients whose tumors decreased in size after treatment (partial response and complete response) are grouped as responders (n = 24), whereas those that had progressive disease are grouped as nonresponders (n = 24). Two patients had stable disease and were removed from the final statistical analysis. The biochemotherapy regimen was administered in 5-day periods at 21-day intervals and included the administration of multiagent chemotherapy, consisting of dacarbazine, cisplatin, vinblastine, and tamoxifen, with the addition of the biological response modifiers, interleukin 2 and IFNα-2b. Patients were accrued through both the John Wayne Cancer Institute and The Angeles Clinic and Research Institute. Human Subjects Institutional Review Board approval was obtained for the purposes of this study at the participating institutions. Signed informed consent was obtained from all patients. Serum samples from 18 healthy donors, which served as controls, were also analyzed.

Fourteen melanoma cell lines established and characterized at the John Wayne Cancer Institute, as previously described, were assessed for *B-RAF*mt (V600E; refs. 24–26). The cell lines were grown in 10% heat-inactivated FCS (Gemini, Calabasas, CA). RPMI 1640 plus penicillin and streptomycin, as previously described, and assessed at early passages (24). DNA was extracted from cells when cultures reached 70% to 80% confluency.

DNA extraction. Blood was collected from patients in TigerTop separation tubes (Fisher Scientific). Serum was immediately separated from blood cells by differential centrifugation at 1,000 × *g* for 15 min, filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh PA), and cryopreserved at −80°C. DNA was isolated from the serum using Qiagen mini-columns (Valencia, CA) according to the manufacturer's instructions, with modifications. DNA was precipitated with 1 μL of Pellet Paint NF coprecipitant (Novagen, Madison, WI) before the proteinase-digested samples were centrifuged. DNA from cell lines was extracted using DNAzol (Molecular Research Center, Cincinnati, OH), as previously described (27). All serum specimens were shown to have DNA.

Oligo design. Briefly, primers were designed to amplify exon 15 of the *B-RAF* gene, including the mutation hotspot (V600E). PNA (Applied Biosystems, Foster City, CA) was designed to clamp the hotspot on the wild-type (wt) template and block the wt template from being amplified by PCR. A fluorescence resonance energy transfer (FRET) dual-labeled LNA probe was designed and synthesized (Proligo, Boulder, CO) to recognize and hybridize at V600E, specifically the T-to-A mutation, as this mutation is the most frequently seen mutation for *B-RAF* at this hotspot (5). A second FRET DNA probe was purchased from Biosource (Camarillo, CA) and synthesized using the adjacent sequences to the LNA probe, avoiding the hotspot, to amplify and estimate the total number of DNA templates, both wild type (V600E) and mutant (V600E), in the PCR reaction. Real-time quantitative PCR for mutation using both the PNA clamp and FRET LNA probe was done

in a separate reaction from the quantitative PCR for total number of templates using the FRET DNA probe.

***Real-time quantitative PCR and quantification of* B-RAF*mt*.** PCR was done using the following primers and probe: *B-RAF*, 5′-CCTC-ACAGTAAAAATAGGTG-3′ (forward), 5′-ATAGCCTCAATTCTTACCA-3′ (reverse), 5′-CTACAGAGAAATCTCGAT-BHQ-1-3′ (LNA), CTACAGTG-AAATCTCG (PNA). The PCR assay was done with the iCycler iQ real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA; Fig. 1). Genomic DNA (20 ng) from serum was amplified using real-time PCR (iCycler) in a 20-μL reaction containing each PCR primer, LNA, PNA, deoxynucleotide triphosphate, $MgCl_2$, PCR buffer, and AmpliTaq Gold Polymerase (Applied Biosystems, Branchburg, NJ). Each PCR reaction was subjected to 55 cycles at 94°C for 60 s, 72°C for 50 s, 53°C for 50 s, and 72°C for 60 s. Each sample was assayed in triplicate with appropriate positive and negative cell line and reagent controls.

We established the MA cell line DNA as the standard for measuring units of V600E *B-RAF*mt target DNA (heterozygous); the amount of target mutant DNA contained in 1 μg/mL MA genomic DNA was arbitrarily established to be 1 unit of *B-RAF*mt. Quantitative PCR results of the samples, generated by iCycler, were compared with this standard to quantify the relative units of *B-RAF*mt in all the samples. All PCR assays for mutant sequence analysis were done in triplicate, and the median was used for data analysis.

Representative *B-RAF*mt V600E and *B-RAF*wt V600E tumors ($n = 4$) were sequenced to confirm the accuracy of the PCR assay, as previously described (5). PCR amplification was done using the following primers for *B-RAF*: 5′-TGTTTTCCTTTACTTACTACACCTCA-3′ (forward) and 5′-AGCATCTCAGGGCCAAAAAT-3′ (reverse). The PCR products were purified with QIAquick PCR Purification kit (Qiagen) and subsequently direct-sequenced at 58°C using Dye Terminator Cycle Sequence Quick Start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. Dye-terminated products were assessed by capillary array electrophoresis on a CEQ8000XL Genetic Analysis System (Beckman Coulter).

***Biochemotherapy response: evaluation of* B-RAF*smt*.** For data analysis, we used the ratio of *B-RAF*smt DNA copies (in units equivalent to V600E mutation copies in μg/mL MA DNA) to total *B-RAF*wt DNA templates (in units equivalent to V600E wt copies in 1 μg/mL DNA) in the reaction as reported results. Mutant DNA copies were calculated by quantitative PCR using a V600E mutant-specific FRET LNA probe with dilution series of MA DNA for the standard curve; total *B-RAF*wt DNA template copies were measured by quantitative PCR with the FRET DNA probe to the V600E region. If the ratio for the post-biochemotherapy serum decreased by one tenth or more when compared with the pre-biochemotherapy serum sample, we determined that the patient "decreased" in *B-RAF*smt; if the ratio increased by ≥10-fold, it was designated as "increased."

Biostatistical analysis. All clinicopathologic factors and *B-RAF*smt frequency were compared by Student's t test and Fisher's exact test. Kaplan-Meier survival curve analysis was used to assess overall and disease-free survival. Univariate analysis of prognostic factors, including age, gender, Eastern Cooperative Oncology Group status, the number of metastatic sites, the site of metastases (soft tissue, lymph nodes, and lung versus other organs), lactate dehydrogenase (LDH) levels, and prior previous treatment (vaccine, chemotherapy, and/or IFN versus no treatment) was assessed. A multivariate analysis using the Cox proportional hazard regression model was also done to evaluate the prognostic significance of *B-RAF*smt when clinical prognostic factors were adjusted. All analyses were done using SAS (SAS/STAT User's Guide, version 8; SAS Institute, Inc., Cary, NC), and tests were two sided with a significance level of <0.05.

Results

B-RAF*mt assay sensitivity*. Using the melanoma cell line MA, shown to have *B-RAF*mt V600E, we did several serial dilution studies to determine the sensitivity of the PNA clamp with the LNA hybridizing probe assay using real-time quantitative PCR. MA DNA in μg/mL was diluted in lymphocyte DNA from normal individuals to simulate an *in vivo* model. The *B-RAF*mt could be detected in 1×10^{-4} unit of MA DNA diluted in 10 units of lymphocyte DNA. However, no *B-RAF*mt (V600E) was

Table 1. Frequency of *B-RAF*smt when compared with known clinical prognostic factors in stage IV melanoma patients receiving biochemotherapy

Clinical factors	*B-RAF*smt ($N = 20$)	*B-RAF*swt ($N = 30$)	*P*
Age			
Mean ± SD	43.4 ± 10.8	45.9 ± 11.8	0.629
≤50	15	19	0.386
>50	5	11	
Gender			
Female	4	8	0.740
Male	16	22	
ECOG			
0-1	8	18	0.166
2	12	12	
No. Met sites			
1-2	10	20	0.239
≥3	10	10	
Met sites			
ST/LN/lung only	2	12	0.021
Other	18	18	
CNS Met	15	28	0.100
LDH			
Mean ± SD	500.4 ± 857.2	295.2 ± 280.7	0.037
≤190	5	17	0.027
>190	15	13	

NOTE: *B-RAF*wt at V600E.
Abbreviations: ECOG, Eastern Cooperative Oncology Group; ST, soft tissue; LN, lymph node; CNS, central nervous system.

Table 2. Univariate analysis of response of biochemotherapy patients

Clinical factors	CR, PR, SD (*N* = 26)	PD (*N* = 24)	*P* (χ^2 test)
Age			
Mean ± SD	46.8 ± 11.9	42.9 ± 10.6	0.341
≤50	15	19	0.104
>50	11	5	
Gender			
Female	5	7	0.411
Male	21	17	
ECOG			
0-1	17	9	0.049
2	9	15	
Met sites			
ST/LN/Lung only	11	3	0.019
Other	15	21	
No. Met sites			
1-2	17	13	0.419
≥3	9	11	
LDH			
Mean ± SD	264.0 ± 259.0	500.0 ± 790.7	0.041
≤190	14	8	0.144
>190	12	16	

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group; ST, soft tissue; LN, lymph node.

detected when 1×10^{-5} unit of MA DNA was diluted in 10 units of lymphocyte DNA. In this series of dilutions, we observed that at >10 units of DNA as a template, the assay will detect amplification of the *B-RAF*wt (V600E) gene as there is only a limited quantity of PNA in each reaction to block amplification of wt DNA. In keeping with this observation, we assessed each sample to estimate the quantity of nonspecific copies to ensure that the DNA templates did not exceed an amount that would result in depletion of the PNA in the reaction based on comparisons with the threshold cycle of the MA DNA dilution series with known units of DNA.

The PNA/LNA PCR assay was subsequently optimized in melanoma cell lines. Fourteen melanoma cell lines were assessed for *B-RAF*mt (V600E), of which 8 (57%) were found to have the *B-RAF*mt. The detection of *B-RAF*mt (V600E) was further validated by sequencing the genomic DNA of the cell lines.

B-RAF*smt of melanoma patients' sera.* Of 103 melanoma patients in the study, including patients treated with biochemotherapy, 38 (37%) patients had *B-RAF*smt detected in their serum. Furthermore, when the patients were divided based on early and advanced stages of disease, *B-RAF*smt was detected in 11 of 34 (32%) early-stage patients (American Joint Committee on Cancer stage I/II) and in 27 of 69 (39%) with metastatic disease (American Joint Committee on Cancer stage III/IV). *B-RAF*smt was not detected in any of the 18 healthy normal donor serum samples.

B-RAF*smt in stage IV melanoma patients.* The frequency of the *B-RAF*smt in 50 stage IV melanoma patients before biochemotherapy was compared with known prognostic factors in melanoma (Table 1). These factors included age, gender, Eastern Cooperative Oncology Group status, the number of metastatic sites, the site of metastases (soft tissue, lymph nodes, and lung versus other organs), LDH levels, and prior previous treatment (vaccine, chemotherapy, and/or IFN versus no treatment). *B-RAF*smt DNA was detected in 20 (42%) patients. The frequency of *B-RAF*mt and *B-RAF*wt DNA was compared with known prognostic factors. Of the factors considered, significant differences were seen in patients who had metastases in soft tissue, lymph nodes, and lung versus other organs ($P < 0.021$) and patients who presented with higher LDH levels ($P < 0.027$; Table 1).

To determine the prognostic significance of *B-RAF*smt in patients after treatment with biochemotherapy, clinicopathologic variables were compared by a univariate analysis (Table 2). Age and gender were not significant predictors of response to treatment. However, Eastern Cooperative Oncology Group status ($P = 0.049$), the site of metastases ($P = 0.019$), and LDH levels ($P = 0.041$) significantly predicted treatment response. The presence of pre-biochemotherapy *B-RAF*smt was not a significant predictor of response to treatment.

Variables found to affect response to biochemotherapy ($P < 0.05$) upon univariate analysis were analyzed by Cox multivariate regression analysis. These variables included age, gender, Eastern Cooperative Oncology Group status, number of metastatic sites, site of metastases (soft tissue, lymph nodes, and lung versus other organs), LDH levels, and prior previous treatment (vaccine, chemotherapy, and/or IFN versus no treatment). Of the factors considered, only Eastern Cooperative Oncology Group status (hazard ratio, 0.24, 95% confidence interval, 0.06-0.98; $P = 0.047$), site of metastases (hazard ratio, 11.5, 95% confidence interval, 1.62-82.5; $P = 0.015$), and previous treatment (chemotherapy and/or IFN versus no treatment: hazard ratio, 0.12, 95% confidence interval, 0.02-0.92; $P = 0.041$) were significant predictors of tumor response to biochemotherapy. However, the presence of pre-biochemotherapy *B-RAF*smt (hazard ratio, 2.2; 95% confidence interval, 0.49-9.80; $P = 0.30$) did not significantly correlate with tumor response to biochemotherapy.

Circulating* B-RAF*smt and survival. Kaplan-Meier curves were developed to determine whether the *B-RAF*smt correlated with overall survival (Fig. 2). A significant difference in overall survival was present between the 20 patients with the *B-RAF*smt

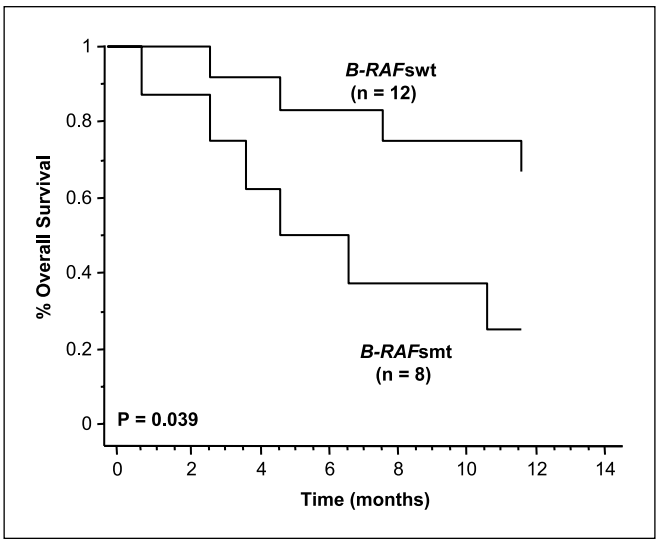

Fig. 2. Kaplan-Meier survival curves of biochemotherapy patients. Correlation of post-biochemotherapy serum *B-RAF* status: *B-RAF*smt and *B-RAF*swt with overall survival (log-rank test, $P = 0.039$).

before biochemotherapy compared with those that did not have the *B-RAF*smt (median, 13 versus 30.6 months, respectively; log-rank, $P = 0.039$).

B-RAF*smt in response to biochemotherapy*. In assessing the change in the detection of circulating *B-RAF*smt in response to biochemotherapy ($N = 48$), serum from 10 of 24 (42%) patients were detected positive for the presence of the *B-RAF*smt in the responder group and 10 of 24 (42%) in the nonresponder group (Fig. 3). However, post-biochemotherapy treatment, circulating *B-RAF*smt was detected in only 1 of the 10 (10%) patients found to have circulating *B-RAF*smt in their pre-biochemotherapy treatment serum in the responder group. In contrast, for the nonresponder group, a statistically significant number of patients (7 of 10, 70%; $P = 0.02$) continued to have circulating *B-RAF*smt in their post-biochemotherapy treatment serum (Table 3). In 30 of the patients, we were able to assess respective melanoma paraffin embedded tissues (primary, metastasis), as previously described (5). Ten of these patients were pre-biochemotherapy *B-RAF*smt positive and had 100% concordance in having *B-RAF*mt (V600E).

In the single patient for the responder group with circulating *B-RAF*smt found in posttreatment serum DNA, we observed that the ratio of the *B-RAF*smt copy number to total serum *B-RAF*wt in serum (*B-RAF*swt) copy number was significantly reduced from that of the pre-biochemotherapy treatment serum sample: 0.11 (pretreatment) to 0.0021 (posttreatment). However, the three patients in the nonresponder group whose post-biochemotherapy treatment serum showed absence of circulating *B-RAF*smt had low mutant to *B-RAF*swt ratios in their pre-biochemotherapy treatment serum. The pretreatment *B-RAF*smt to *B-RAF*swt DNA ratio ranged from 1.5×10^{-3} to 9.0×10^{-6} in these three patients, whereas the ratio was substantially higher in the other pre-biochemotherapy treatment serum samples.

In comparing the pre-biochemotherapy treatment sera to the post-biochemotherapy treatment sera, we observed that the *B-RAF*smt DNA ratio decreased in all 24 patients in the responder group and 20 of 24 patients in the nonresponder group. Of the remaining patients in the nonresponder group, one patient was found to have increased, and three patients had "no remarkable changes" in the *B-RAF*smt DNA ratio. This observation may have been related to the possibility that circulating *B-RAF*smt was not detected due to insufficient amounts of DNA.

Discussion

The frequency of *B-RAF*mt (V600E and other sites) in patients with metastatic melanoma has been reported to be >55% (28–31). *B-RAF*mt has been suggested to contribute to the development of melanoma; however, this topic has been under debate. Primary melanomas of different types vary in *B-RAF*mt frequency. In the biochemotherapy group studied, 43 of the 50 patient (93%) primaries identified were of cutaneous origin. The importance of mutations in both the *N-RAS* and

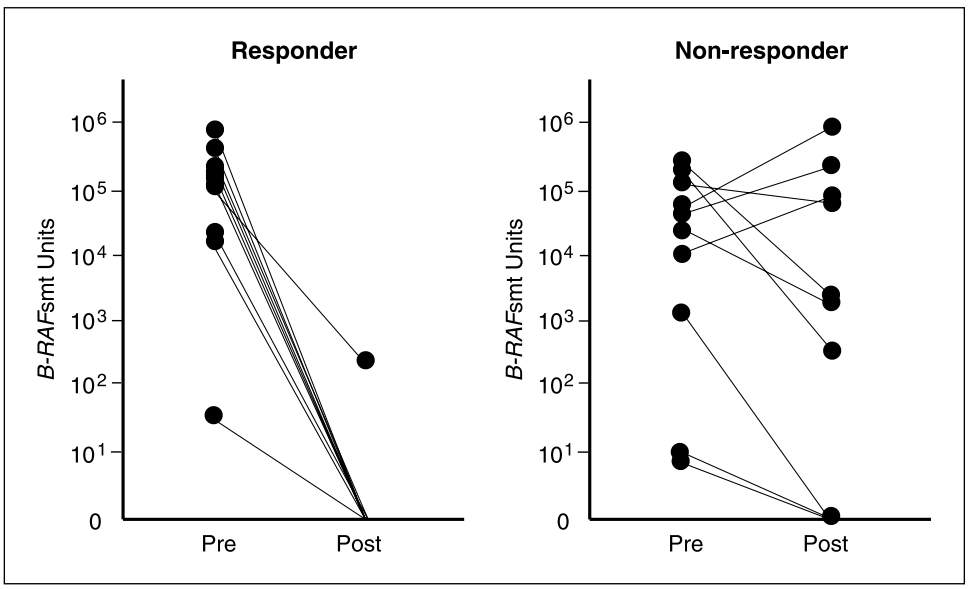

Fig. 3. DNA concentration of circulating *B-RAF*smt in serum of responders and nonresponders after biochemotherapy treatment.

Table 3. *B-RAF*smt status in response to biochemotherapy

	Response, *n* (%)	Nonresponse, *n* (%)
Pre-*B-RAF*smt/post-*B-RAF*smt	1 (10)	7 (70)
Pre-*B-RAF*smt/post-*B-RAF*swt	9 (90)	3 (30)
Total	10 (100)	10 (100)

NOTE: Comparison of response to nonresponse ($P = 0.02$).

B-RAF genes in dysplastic nevi and melanomas has been of considerable interest in that deregulation of the RAS-RAF-MEK-ERK pathway may be important in melanoma progression (2, 14). Because the *B-RAF*mt (V600E) has been shown to occur frequently in metastatic melanoma, it is important to determine if *B-RAF*mt V600E can be used to detect patients with metastatic melanoma and identify which patients would potentially be more responsive to specific adjuvant therapies.

In this study, we used a highly specific assay that recognizes a single base pair mismatch to detect the *B-RAF* mutation at V600E. This is the first study showing ability to detect *B-RAF*smt in melanoma patients and potential clinical utility of predicting response to biochemotherapy. In stage IV patients who underwent biochemotherapy, a significant number of patients ($P = 0.02$) who did not respond to biochemotherapy continued to have circulating *B-RAF*smt after the completion of treatment. Moreover, only one patient with a clinical response to biochemotherapy was found to have circulating *B-RAF*smt. The presence of *B-RAF*smt in these patients indicated a lack of clinical response. The explanation for the lack of *B-RAF*smt in responding patients is that tumors responding to biochemotherapy undergo apoptosis, thus inducing DNA to breakdown into small fragments, which, when shed into body fluids, get rapidly cleared away. In nonresponding patients, DNA can be released by tumor cell turnover, physical disruption of circulating tumor cells, and/or from tumor necrosis. The DNA released from these processes may not have gone through apoptosis processes, thus maintaining the DNA integrity and is released as longer sized fragments.

Although presence of the *B-RAF*smt did not significantly correlate with treatment response when compared with other known prognostic factors, such as location of metastases, LDH levels, and prior treatment, the presence of post-biochemotherapy circulating *B-RAF*smt in patients did correlate with significantly poorer outcomes, such as decreased overall survival.

This pilot study shows the potential clinical utility of monitoring patients with metastatic melanoma receiving therapy. Because studies have shown the frequency of *B-RAF*mt (V600E) in metastatic melanoma tissue to be higher than in primary tumors, the serum assay may also be useful in patient follow-up for monitoring disease progression (13, 32–34). There are reports suggesting that *B-RAF*mt (V600E) may be important in disease progression and may potentially be of prognostic utility (29, 32, 34). Our current findings showed no significant correlation with known clinical variables that have been shown to affect outcome.

In conclusion, our findings confirm that the presence of the *B-RAF*smt in circulating DNA in serum may have clinical utility in predicting tumor response and disease outcome. Although *B-RAF*smt was not associated with other markers of disease progression, our study did show that the presence of the mutation confers poor outcomes with significantly lower overall survival. The *raf* kinase inhibitor sorafenib (BAY 43-9006), which inhibits melanoma and other cancers by targeting the RAF/MEK/ERK pathway (3, 35, 36), has been Food and Drug Administration approved for renal cell carcinoma. BAY 43-9006 used alone has been disappointing in melanoma patients. The combination of BAY 43-9006 with other drugs may have benefits to melanoma patients. The detection of circulating *B-RAF*smt before initiation of therapy may be very useful in monitoring treatment response to RAF/MEK/ERK pathway–targeted drugs.

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