

Quantitative Cell-Free Circulating $BRAF^{V600E}$ Mutation Analysis by Use of Droplet Digital PCR in the Follow-up of Patients with Melanoma Being Treated with BRAF Inhibitors

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BACKGROUND: Around 50% of cutaneous melanomas harbor the $BRAF^{V600E}$ mutation and can be treated with BRAF inhibitors. DNA carrying this mutation can be released into circulation as cell-free $BRAF^{V600E}$ ($cfBRAF^{V600E}$). Droplet digital PCR (ddPCR) is an analytically sensitive technique for quantifying small concentrations of DNA. We studied the plasma concentrations of $cfBRAF^{V600E}$ by ddPCR in patients with melanoma during therapy with BRAF inhibitors.

METHODS: Plasma concentrations of $cfBRAF^{V600E}$ were measured in 8 controls and 20 patients with advanced melanoma having the $BRAF^{V600E}$ mutation during treatment with BRAF inhibitors at baseline, first month, best response, and progression.

RESULTS: The $BRAF^{V600E}$ mutation was detected by ddPCR even at a fractional abundance of 0.005% in the wild-type gene. Agreement between tumor tissue $BRAF^{V600E}$ and plasma $cfBRAF^{V600E}$ was 84.3%. Baseline $cfBRAF^{V600E}$ correlated with tumor burden ($r = 0.742$, $P < 0.001$). $cfBRAF^{V600E}$ concentrations decreased significantly at the first month of therapy (basal median, 216 copies/mL; Q1–Q3, 27–647 copies/mL; first response median, 0 copies/mL; Q1–Q3, 0–49 copies/mL; $P < 0.01$) and at the moment of best response (median, 0 copies/mL; Q1–Q3, 0–33 copies/mL; $P < 0.01$). At progression, there was a significant increase in the concentration of $cfBRAF^{V600E}$ compared with best response (median, 115 copies/mL; Q1–Q3, 3–707 copies/mL; $P = 0.013$). Lower concentrations of basal $cfBRAF^{V600E}$ were significantly associated with longer overall survival and progression-free survival (27.7

months and 9 months, respectively) than higher basal concentrations (8.6 months and 3 months, $P < 0.001$ and $P = 0.024$, respectively).

CONCLUSIONS: $cfBRAF^{V600E}$ quantification in plasma by ddPCR is useful as a follow-up to treatment response in patients with advanced melanoma.

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Cutaneous melanoma is a tumor with increasing worldwide incidence, which in advanced stages is among the most aggressive and treatment-resistant human cancers. Mutations in $BRAF$ (B-Raf proto-oncogene serine/threonine kinase)⁵ are present in >50% of cutaneous melanomas (1), and >80% of these correspond to the T1799A mutation that results in the substitution of valine to glutamic acid at codon 600 ($BRAF^{V600E}$) (1, 2). This produces a constitutive activation of BRAF, which increases the Raf–mitogen-activated protein kinase kinase (MEK)⁶ signaling pathway that controls proliferation, cell survival, and invasion (3). Tumors bearing $BRAF^{V600E}$ mutations are sensitive to therapy with BRAF inhibitors (iBRAF) (4), which have shown to improve survival in these patients (5, 6). As a consequence, $BRAF$ mutation analysis in tumor biopsy is becoming a routine to select patients that could benefit from this therapy (7).

Increased concentrations of circulating cell-free DNA (cfDNA) have been found in cancer patients (8). Apoptotic and necrotic cancer cells are a main source of cfDNA, harboring the same genetic alterations present in the corresponding tumor (9). Specifically, cfDNA with mutations in $PIK3CA$

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⁵ Human genes: $BRAF$, B-Raf proto-oncogene serine/threonine kinase; $PIK3CA$, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α ; $EGFR$, epidermal growth factor receptor; $KRAS$, Kirsten rat sarcoma viral oncogene homolog; $NRAS$, neuroblastoma RAS viral (v-ras) oncogene homolog.

⁶ Nonstandard abbreviations: MEK, mitogen-activated protein kinase kinase; iBRAF, BRAF inhibitor; cfDNA, cell-free DNA; ddPCR, droplet digital PCR; RECIST, Response Evaluation Criteria in Solid Tumors; LDH, lactate dehydrogenase; MIA, melanoma inhibitory activity; OS, overall survival.

(phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α) (10), *EGFR* (epidermal growth factor receptor) (11), *KRAS* (Kirsten rat sarcoma viral oncogene homolog) (12), and *BRAF* (13) has been detected in cancer patients. Determination of mutations in cfDNA could become a useful tool to perform sequential evaluations of tumor mutation status in blood (“liquid biopsy”), avoiding the need for multiple and sequential biopsies (14, 15). Particularly, circulating cell-free *BRAF*^{V600E} (cf*BRAF*^{V600E}) has been reported in thyroid cancer (16) and melanoma (17), although there are few studies supporting its use in the diagnosis, prognosis, and follow-up (13, 16).

A crucial issue related to the detection of mutations in blood is that most cfDNA is wild-type, and the tumor-derived mutant DNA fraction could be <0.01% (18). Most of the PCR methods developed to detect the *BRAF*^{V600E} mutation are limited by the presence of a high proportion of wild-type sequences, resulting in detection limits on the order of 0.1%–2% of mutant DNA in a pool of wild-type *BRAF* (17, 19), so a very analytically sensitive quantification method is needed. Droplet digital PCR (ddPCR) is a suitable technique for measuring circulating cell-free nucleic acids because it can detect and quantify very small amounts of mutated DNA without the requirement of a calibration curve (14, 20). ddPCR has several advantages compared with quantitative RT-PCR, such as being more precise, better at detecting rare genetic variants, and less susceptible to inhibitors (14, 21). These advantages make this technique suitable for analysis of tumor mutations in blood. Indeed, digital PCR has already been used to detect *BRAF*-mutated DNA in blood from advanced melanoma patients (11).

The monitoring of treatment responses is essential to determine the benefit of new therapies to avoid prolonged use of ineffective and potentially toxic treatments. There is an unmet need for biomarkers for measuring the tumor burden in melanoma with high diagnostic sensitivity and specificity as a proper surrogate of tumor response (22, 23). The aims of the present work were to analyze the changes in the concentrations of cf*BRAF*^{V600E} in blood by ddPCR in patients with advanced melanoma being treated with iBRAF and to correlate the changes with the clinical evolution of the disease.

Materials and Methods

CELLS

We obtained the HT29 human colon carcinoma cell line from American Type Culture Collection. The cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere with RPMI-1640 medium supplemented with 10% heat

Table 1. Patient baseline characteristics (n = 20).

Variable	Overall	Treatment
Age (years)	50 (12)	
Sex		
Male	13 (65)	3 D/10 V ^a
Female	7 (35)	2 D/5 V
Stage		
Unresectable IIIc	1 (5)	0 D/1 V
IVa	5 (25)	2 D/3 V
IVb	1 (5)	0 D/1 V
IVc	13 (65)	3 D/10 V
Primary site		
Cutaneous	16 (80)	4 D/12 V
Mucosal	0 (0)	0 D/0 V
Uveal	0 (0)	0 D/0 V
Acral	2 (10)	0 D/2 V
Unknown	2 (10)	1 D/1 V

Data are mean (SD) or n (%).
^a D, dabrafenib; V, vemurafenib.

inactivated fetal bovine serum, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 2 mmol/L L-glutamine (Gibco).

PATIENTS AND TREATMENT

From April 2011 to May 2014, we treated 20 patients with stage IIIc and IV melanoma and a positive test for the *BRAF*^{V600} mutation in a tumor biopsy with the iBRAF dabrafenib or vemurafenib (Table 1). We evaluated tumor response by physical examination and imaging studies using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, with the modification to measure all lesions, not just the target lesions (24). Best response was defined as the best objective response (stable disease, partial response, complete response, or progressive disease) assessed between the first day of treatment to progression, death, or last follow-up. Eight healthy subjects were anonymized and studied as control samples. The protocol for the study was approved by the ethics committee (reference 111/2010), and all patients signed written informed consent.

V600 MUTATION ANALYSIS IN TUMOR BIOPSIES

Before treatment, the presence of BRAF mutations was confirmed in tumor biopsies from all patients. DNA from tumor cells was isolated with the cobas DNA Sample Preparation Kit (Roche Molecular Systems) (25). We determined the *BRAF*^{V600} mutation by real-time PCR assay with the kit cobas 4800 BRAF V600 Mutation Test (Roche Molecular Systems) according to the manufac-

turer's instructions. This method detects predominantly the V600E mutation (26).

SAMPLE COLLECTION

Blood samples (10 mL) were collected in vacutainer tubes (Becton Dickinson) both with and without EDTA at baseline and sequentially at each visit. Blood samples were centrifuged within 1 h after collection at 2000g for 10 min and stored at -80°C until analysis. We analyzed the samples corresponding to the dates of baseline, 1 month after starting treatment, best response, and progressive disease.

Blood (5 mL) was collected from a healthy volunteer in EDTA-containing vacutainer tubes and assayed without centrifugation the same day.

SERUM ASSAYS

We analyzed lactate dehydrogenase (LDH) activity and melanoma inhibitory activity (MIA) and S100 concentrations in serum samples according to the manufacturer's instructions. LDH was analyzed with a kit from Roche on a Modular Analytics P800 analyzer (Roche), MIA with a quantitative ELISA kit (Roche), and S100 with an electrochemiluminescence assay (Roche) on a Modular E170 analyzer (Roche). The upper reference limits were S100, 0.1 $\mu\text{g/L}$; MIA, 9 $\mu\text{g/L}$; and LDH, 436 U/L.

DNA EXTRACTION

We extracted genomic DNA with the QIAamp DNA Blood Mini Kit (Qiagen) and cfDNA with the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's protocol. DNA quantification was performed in a NanoDrop (Thermo Scientific). DNA samples were kept at -80°C until used (<1 month).

cfDNA QUANTIFICATION BY ddPCR

In ddPCR, the PCR reaction mixture is partitioned into thousands of droplets so that each compartment contains either 1 or 0 molecules of target and background DNA (20). The droplets then undergo PCR amplification, and a fluorescence signal is produced in each droplet with the target molecule. Quantification of the number of target DNA molecules in the reaction is achieved by counting the number of positive and negative droplets.

ddPCR assays were performed with the PrimePCR™ ddPCR™ Mutation Detection Assay kit (Bio-Rad Laboratories) that used an amplicon of 91 nt (manufacturer's information; see Supplemental Table 1, which accompanies the online version of this article at <http://www.clinchem.org/content/vol61/issue1>). The cfDNA concentrations were normalized to the precise concentration of a genomic DNA sample and amplified with the same primer set in both cases. We used DNA from the HT29 cell line as positive control and from

leukocytes from a donor as negative control. Background was analyzed with water instead of DNA. All samples were analyzed at least in duplicate. Amplifications were carried out in a reaction volume of 20 μL on a QX100 Droplet Digital PCR System (Bio-Rad). The 20- μL PCR mix was composed of 10 μL Bio-Rad Super mix TaqMan, 1 μL of each (target and reference) amplification primer/probe mix (450 and 250 nmol/L, respectively), and 8 μL cfDNA extracted. The thermal cycling comprised an initial denaturing and polymerase hot-start activating step of 10 min at 95°C , followed by 40 repeated cycles of 95°C for 30 s and 55°C for 60 s. Results were analyzed with Quantasoft v.1.3.2 software (Bio-Rad) and reported as copies per milliliter of plasma.

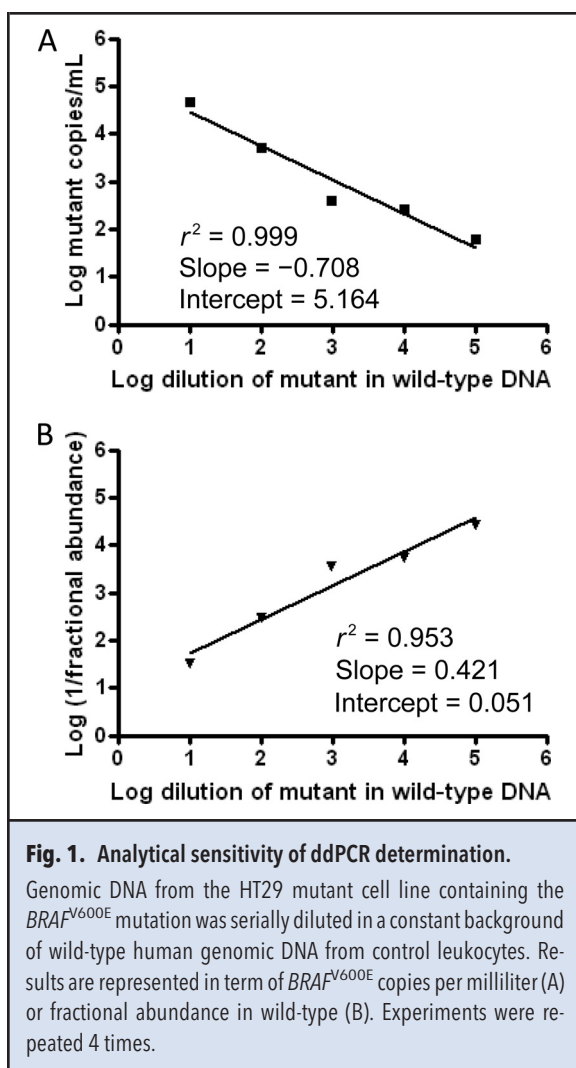
STATISTICAL ANALYSIS

Results were expressed as median and 25th/75th percentiles after determining their nongaussian distribution with Kolmogorov–Smirnov and Shapiro–Wilk tests. We applied nonparametric Kruskal–Wallis and Mann–Whitney *U*-test tests for comparison between groups. Wilcoxon test was performed to compare changes in the concentrations during treatment. Correlation analyses were performed with the Spearman rank correlation coefficient. Progression-free survival and overall survival (OS) were measured from the time of iBRAF treatment initiation to the time of progression, death, or last follow-up and were analyzed by the Kaplan–Meier method and compared by log-rank and Breslow tests. A 2-tailed *P* value ≤ 0.05 was considered to be statistically significant. Statistical analysis was performed with IBM SPSS 20.

Results

VALIDATION OF THE DIGITAL PCR FOR ANALYSIS OF cf*BRAF*^{V600E} MUTATION ASSAY IN PLASMA

DNA samples used for assay validation were from the human colon cancer cell line HT29, which harbors the *BRAF*^{V600E} mutation. There was a clear difference between the signal produced by *BRAF*^{V600E} mutation and by wild-type DNA (see online Supplemental Fig. 1). In addition, we only detected wild-type *BRAF* sequences and no mutant copies in 0.75 ng/ μL wild-type DNA obtained from leukocytes ($n = 5$). Analysis of a blank sample without DNA ($n = 5$) resulted in a lack of detection of both wild-type and mutant copies. To assess the analytical sensitivity of the assay measuring the *BRAF*^{V600E} mutation, we performed serial dilutions of DNA from the HT29 cell line into DNA from a wild-type source (16 ng/ μL to 1.6 pg/ μL). The mutation could be detected by ddPCR even at a dilution of 0.001% (Fig. 1) with a fractional abundance of 0.005%, and the linearity of the assay was maintained ($R^2 = 0.999$). For



these reasons, the limit of detection was established as 1 copy of mutant DNA/mL.

We analyzed the presence of *cfBRAF*^{V600E} in the blood of 8 healthy donors. In 1 control of 8, we detected low concentrations of *cfBRAF*^{V600E} (12 copies/mL), and in the other 7 only wild-type sequences of *cfBRAF*^{V600E} were detected.

BASAL *cfBRAF*^{V600E}

We studied 20 patients with advanced melanoma treated in our institution with the iBRAF dabrafenib or vemurafenib. Clinical features are summarized in Table 1. From these patients, 19 plasma samples were available for quantification of the number of copies per milliliter of *cfBRAF*^{V600E} at baseline. The percentage of patients with *cfBRAF*^{V600E} was 84.3% (median, 216 copies/mL; Q1–Q3, 27–647 copies/mL). In contrast, no mutant copies were detected in 3 patients that harbored the *BRAF*^{V600E}

mutation in the tumor tissue, as determined by the Sanger method (data not shown). These 3 patients, at the moment of the *cfBRAF*^{V600E} determination, had a low tumor burden (40, 38, and 16 mm, respectively). Also, the fractional abundance of *cfBRAF*^{V600E} was highly variable, with a median of 2.97% (Q1–Q3, 0.22%–9.8%).

There was a significant relationship between the number of mutant copies per milliliter and the tumor burden ($r = 0.742$; $P < 0.001$) (Fig. 2A). We also found a correlation between the concentrations of *cfBRAF*^{V600E} and the tumor markers MIA ($r = 0.708$, $P = 0.001$) and S100 ($r = 0.543$, $P = 0.02$) and the enzyme activity of LDH ($r = 0.617$, $P = 0.007$) (Fig. 2B).

ASSESSMENT OF *cfBRAF*^{V600E} DURING THERAPY

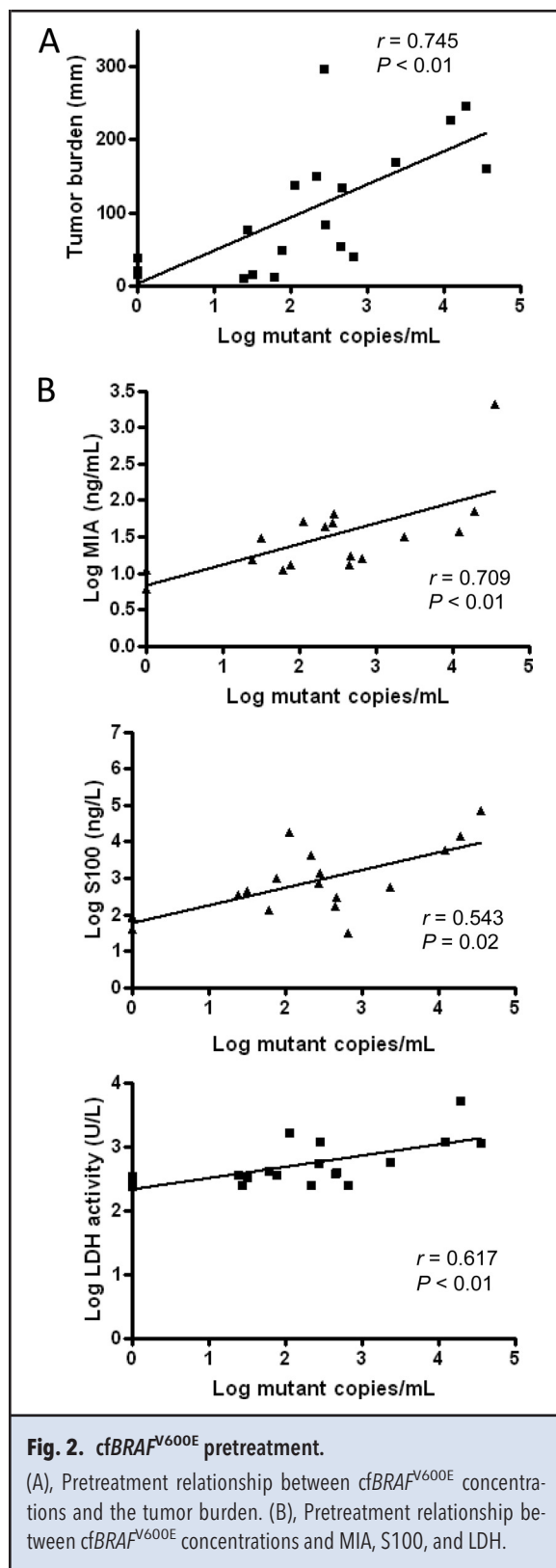
We followed the evolution of the patients from the beginning of treatment, during the development of clinical responses, and until disease progression or death. Eighteen (90%) of 20 treated patients responded to the iBRAF treatment, and at the moment of best response, 5 patients had complete response, whereas the other 13 patients had a partial response. However, 16 (80%) of these 18 responders relapsed during follow-up, with a median duration of response of 4.5 months.

At the first month of therapy, the concentration of *cfBRAF*^{V600E} in plasma decreased significantly (median, 0 copies/mL; Q1–Q3, 0–49 copies/mL; $P < 0.01$ vs basal) (Fig. 3A). The decrease was maintained at the moment of the best response (median, 0 copies/mL; Q1–Q3, 0–33 copies/mL; $P < 0.01$ vs basal), but 40% of these patients had detectable *cfBRAF*^{V600E} mutations in blood at that moment. Interestingly, 4 of 5 patients with complete response were negative for circulating *cfBRAF*^{V600E} mutation.

Sixteen plasma samples from patients with progression were available for quantification. Twelve patients (75%) had detectable concentrations of circulating *cfBRAF*^{V600E} mutations (median, 115 copies/mL; Q1–Q3, 3–707 copies/mL; $P = 0.013$ vs best response) (Fig. 3A). Furthermore, 6 of these patients had undetectable concentrations of *cfBRAF*^{V600E} mutations at the moment of best response, but at progression they had detectable circulating DNA with *BRAF*^{V600E} mutations (Fig. 3B; online Supplemental Fig. 2). During treatment, the number of copies per milliliter of *cfBRAF*^{V600E} mutations in plasma did not correlate with LDH, S-100, or MIA concentrations measured at the same points.

PROGNOSTIC SIGNIFICANCE OF *cfBRAF*^{V600E}

Patients with ≥ 2 -year overall survival had lower number of copies per milliliter of *cfBRAF*^{V600E} mutations than patients with < 2 years overall survival (> 2 -year OS, 27 copies/mL; Q1–Q3, 0–76 copies/mL; < 2 -year OS, 478

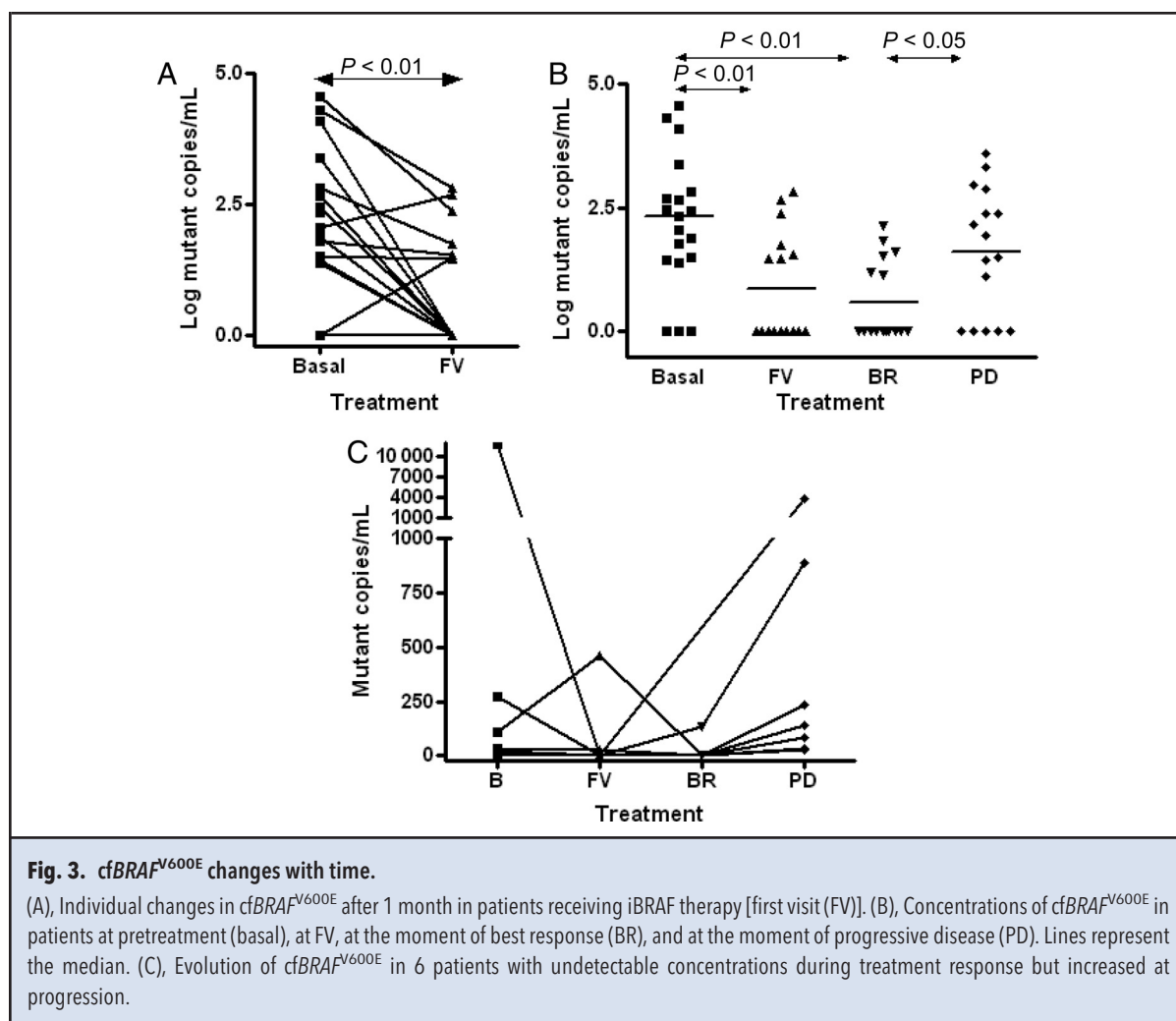


copies/mL; Q1–Q3, 138–9537 copies/mL; $P = 0.01$) (Fig. 4A). Furthermore, considering 216 cfDNA mutant copies/mL as the cutoff, those patients with a lower number of basal $cfBRAF^{V600E}$ copies per milliliter had a mean overall survival of 27.7 months (95% CI, 21–34), longer than those with higher basal $cfBRAF^{V600E}$ copies per milliliter (mean, 8.6 months; 95% CI, 4–13; $P < 0.001$) (Fig. 4B). Also, these patients had a better progression-free survival (median, 9 months; 95% CI, 0.2–18) compared with those with higher basal $cfBRAF^{V600E}$ copies/mL (median, 3 months; 95% CI, 1–5; $P = 0.024$). Similar results were observed when we studied the presence of circulating mutation in terms of fractional abundance instead of copies per milliliter (data not shown). Also, patients with cfDNA negative for $cfBRAF^{V600E}$ at the moment of best response had longer survival, although the difference was not significant.

Discussion

An important problem relating to detecting mutation in blood is that most cfDNA is wild-type, and the tumor-derived mutant DNA fraction in cfDNA can be $<0.01\%$ (18, 20). With reproducible results, we could detect the $BRAF^{V600E}$ mutation at a lower dilution (Fig. 1), confirming that ddPCR is a reliable method to detect the $cfBRAF^{V600E}$ mutation in blood, is more analytically sensitive than other methods (17), and has a specificity similar to that reported by others (19). We have reported our data in copies per milliliter (10, 11), although some authors have shown their results as percent of reactions that are mutant (12). We agree with Oxnard et al. (11) that the use of copies per milliliter could be more appropriate for low abundance of DNA, which occurs in plasma. Those authors used a threshold of 0.5 copies/mL for a positive result, and they observed a diagnostic sensitivity of 87.5%, similar to that reported by us.

In this study, we found a very high percentage of agreement of positivity in the analysis for $BRAF^{V600E}$ mutation between tumor tissue and plasma, similar to that previously reported (27). These days, the selection of patients with melanoma to receive iBRAF treatment is based on the analysis of the $BRAF^{V600}$ mutation in the tumor tissue, but it has been claimed that there is 13.5%–15% of discordance in the mutational status between the primary tumor and paired metastasis (2, 28, 29). The absence of $BRAF^{V600}$ mutation in 1 tumor biopsy sample may not be a definitive result, and the analysis of other biopsies at the same time from different tumor lesions is not practical. Therefore, the high analytical sensitivity of this method and the fact that cfDNA can reflect the BRAF status in any lesion of the body suggest that the analysis of $cfBRAF^{V600E}$ mutation in blood could help to select melanoma patients for iBRAF therapy (29). It has



been proposed in differentiated thyroid carcinoma that data from $cfBRAF^{V600E}$ can complement the information obtained from cytological analysis, providing more complete information (16). However, we should consider that this mutation could be present in both malignant (1, 16) and benign (30) situations, affecting the diagnostic specificity of the $cfBRAF^{V600E}$ analysis.

We observed a relationship between basal $cfBRAF^{V600E}$ and tumor burden, which is likely a consequence of DNA released into circulation from tumor cells. Interestingly, a higher concentration of $cfBRAF^{V600E}$ was associated with a worse clinical outcome. In contrast, *BRAF* mutations detected in tumor tissue do not correlate with disease outcome (31). Other circulating tumor markers, such as LDH, MIA, and S100B, have been proposed to be of use in the follow-up of the patients (22, 23), and these 3 tumor markers also correlated with $cfBRAF^{V600E}$ at baseline. Additionally,

because this DNA mutation is a requisite for therapy with iBRAF, its analysis in blood could be of value as a surrogate of tumor burden during the treatment of melanoma $BRAF^{V600E}$ patients. The changes observed in the number of copies per milliliter are in the logarithmic scale and are very clear in our series of patients. A decrease of the $cfBRAF^{V600E}$ concentrations detected indicates the efficacy of the therapy, since it could be related to the destruction of the tumor cells and a rapid clearance of the mutant DNA (16). In the present work, treatment induced a reduction in the number of mutant copies, and in some cases the mutation was not even detected in plasma. Also very interesting was the increased concentration of mutant copies observed following disease progression that reflects the state of a secondary resistance to the treatment, in which tumor cells most likely have evaded the blockade of iBRAF, increasing the number of melanoma $BRAF^{V600E}$ cells (see online Supplemental Fig. 2) (32).

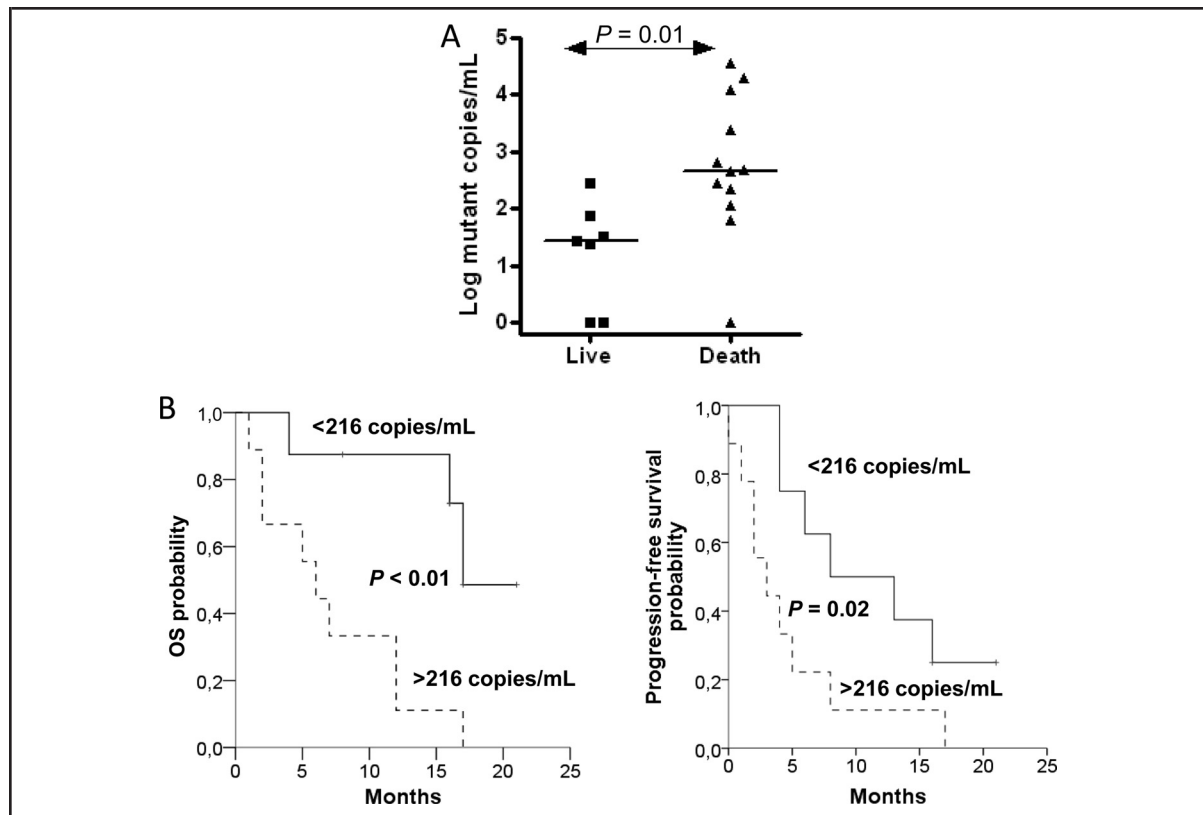


Fig. 4. cfBRAF^{V600E} and survival.

(A), Concentrations of basal cfBRAF^{V600E} in patients with ≥ 2 -year OS or < 2 -year OS. (B), Kaplan-Meier plot representing OS (left) and progression free survival (right) probabilities for patients with advanced melanoma according to basal cfBRAF^{V600E}. Cutoff for cfBRAF^{V600E} concentrations = 216 copies/mL.

By mutation analysis in blood, we could obtain more dynamic information related to the mutational profile of the tumor. Particularly, ddPCR could be a method for monitoring the prevalence of tumor clones harboring the *BRAF*^{V600E} mutation in the body, where an increase in cfBRAF^{V600E} concentrations could be an analytical sign that the therapy is inefficient. Additionally, simultaneous blood analysis of other mutations different from *BRAF*^{V600E} at the moment of progression could lead to an understanding of the mechanism of secondary resistance to iBRAF treatment (12). It has been shown recently that the acquisition of *NRAS* [neuroblastoma RAS viral (v-ras) oncogene homolog]-activating somatic mutations is a molecular mechanism of resistance to iBRAF treatment in patients harboring the *BRAF*^{V600E} mutation (33).

In conclusion, the results from our study show that cfBRAF^{V600E} analysis by ddPCR of blood from patients with melanoma has potential value for identifying pa-

tients that could benefit of treatment with iBRAF and for monitoring response to treatment.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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