Title:

Quantitative cell-free circulating *BRAF*^{V600E} mutation analysis using droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors

Running head:

Plasma cell-free *BRAF*^{V600E} analysis in melanoma

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List of abbreviations:

BRAF: B-Raf proto-oncogene serine/threonine kinase; iBRAF: BRAF inhibitor; cfDNA: cell-free DNA; cf*BRAF*^{V600E}: cell-free BRAF^{V600E}; ddPCR: Droplet digital PCR; RECIST: Response Evaluation Criteria in Solid Tumors; OS: Overall survival.

Abstract

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} (cf*BRAF*^{V600E}). Droplet digital PCR (ddPCR) is an analytically sensitive technique for quantifying small concentrations of DNA. We studied the plasma concentrations of cf*BRAF*^{V600E} by ddPCR in patients with melanoma during therapy with BRAF inhibitors.

Methods: Plasma concentrations of cf*BRAF*^{V600E} were determined in eight controls and twenty 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 cf*BRAF*^{V600E} was 84.3 %. Baseline cf*BRAF*^{V600E} correlated with tumor burden ($r=0.742$; $p<0.001$). cf*BRAF*^{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 cf*BRAF*^{V600E} as compared with best response (median: 115 copies/mL; Q1-Q3: 3-707 copies/mL; $p=0.013$). Lower concentrations of basal cf*BRAF*^{V600E} were significantly associated with longer overall survival and progression-free survival (27.7 months and 9 months) than higher basal concentrations (8.6 months and 3 months, $p<0.001$ and $p=0.024$ respectively).

Conclusions: cf*BRAF*^{V600E} quantification in plasma by ddPCR is useful to follow-up treatment response in patients with advanced melanoma.

Introduction

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 the B-Raf proto-oncogene serine/threonine kinase (*BRAF*) are present in more than 50% of cutaneous melanomas (1), and more than 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/MEK signaling pathway that controls proliferation, cell survival and invasion (3). Tumors bearing $BRAF^{V600}$ 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 one of the main sources of cfDNA, harboring the same genetic alterations present in the corresponding tumor (9). Specifically, cfDNA with mutations in *PIK3CA* (10), *EGFR* (11), *KRAS* (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 lower than 0.01% (18). Most of the PCR methods developed to detect $BRAF^{V600E}$ mutation are limited by presence of

a high proportion of wild type sequences resulting in detection limits in the order of 0.1%-2% of mutant DNA in a pool of wild type *BRAF* (17, 19) so an analytically very sensitive quantification method is needed. Droplet digital PCR (ddPCR) is a suitable technique for measuring circulating cell-free nucleic acids since it can detect and quantify very small amounts of mutated DNA without the requirement of a calibration curve (14, 20). DdPCR has several advantages as 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 very 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.

Material and Methods

Cells

HT29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured at 37ºC in a 5% CO_2 humidified atmosphere with RPMI-1640 medium supplemented with 10% heat inactivated Fetal Bovine Serum, 50 U/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine (Gibco).

Patients and treatment

Twenty patients with stage IIIc and IV melanoma and a positive test for the *BRAF*^{V600} mutation in a tumor biopsy were treated with the iBRAF dabrafenib or vemurafenib, from April 2011 to May 2014 (Table 1). Evaluation of tumor response was assessed 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 our 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 using the Cobas DNA Sample Preparation Kit (Roche Molecular Systems) (25). *BRAF V600* mutation was determined by real-time polymerase-chain-reaction assay with the kit Cobas 4800 *BRAF V600* Mutation Test (Roche Molecular Systems) according to manufacturer's instructions. This method detects predominantly the V600E mutation (26).

Sample collection

Blood samples (10 mL) were collected in both EDTA-containing or without-additive vacutainer tubes (Becton and Dickinson) at baseline and sequentially at each visit. Blood samples were centrifuged within 1 h after collection at 2,000 x g for 10 min and stored at -80ºC until analysis. Analysis were performed in samples corresponding to the

dates of baseline, one month after starting treatment (FR), best response (BR) and progressive disease (PD).

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

Serum assays

Lactate dehydrogenase (LDH) activity and melanoma inhibitory activity (MIA) and S100 concentrations in serum samples were analyzed following manufacturer's instructions. LDH was analyzed using a kit from Roche on a Modular Analytics P800 analyzer (Roche). MIA was determined by a quantitative ELISA kit (Roche) and S100 was analyzed by an electrochemiluminiscence assay (Roche) in a Modular E170 analyzer (Roche). The upper reference limits were S100=0.1 µg/L; MIA=9 µg/L; and LDH = 436 U/L.

DNA extraction

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and cfDNA was extracted using 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 (less than one month).

cfDNA quantification by droplet digital PCR

Droplet digital PCR consists on the partition of the PCR reaction mixture 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 using the PrimePCR™ ddPCR™ Mutation Detection Assay kit (Bio-Rad Laboratories) that used an amplicon of 91nt (manufacturer's information, Supplemental Table 1). The cfDNA concentrations were normalized to the precise concentration of a genomic DNA sample and amplified using the same primer set in both cases. DNA from HT29 cell line was used as positive control and from leukocytes from a donor as negative control. Background was analyzed using 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 of Bio-Rad Super mix TaqMan, 1 µl of each (target and reference) amplification primer/probe mix (450 nM/250 nM, respectively), and 8 µl of 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 using Quantasoft v.1.3.2 software (Bio-Rad) and reported as copies/mL of plasma.

Satistical analysis

Results were expressed as median and 25th-75th percentiles after determining their non-Gaussian distribution with the Kolmogorov-Smirnov and Shapiro-Wilks tests. The non-parametric Kruskal-Wallis and Mann-Whitney U-test tests were applied for comparison between groups. Wilcoxon test was performed to compare changes in the concentrations during treatment. Correlation analyses were performed using the Spearman's Rank Correlation Coefficient. Progression-free survival and overall survival were respectively measured from the time of iBRAF treatment initiation to the time of progression, death or last follow-up and they were analyzed by the Kaplan-Meier method and compared by

the log-rank and Breslow tests. A two-tailed *P*-value ≤ 0.05 was considered to be statistically significant. Statistical analysis was performed with IBM SPSS 20 (Somers, NY, USA).

Results

Validation of the digital PCR for analysis of cfBRAF^{V600E} mutation assay in plasma

DNA samples used for assay validation were from the human colon cancer cell line HT-29, which harbors the *BRAF*^{V600E} mutation. There was a clear difference between the signal produced by *BRAF*^{V600E} mutation and by wild type DNA (Supplemental Figure 1). In addition, it was only detected wild type *BRAF* sequences and no mutant copies in 0.75 ng/µL of 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 *BRAF^{V600E}* mutation, we performed serial dilutions of DNA from HT29 cell line into DNA from a wild type source (range from 16 ng/µL to 1.6 pg/µL). The mutation could be detected by ddPCR even at a dilution of 0.001% (Figure 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 one copy of mutant DNA/mL.

We analyzed the presence of cf*BRAF^{V600E}* in the blood of 8 healthy donors. In one control out of eight we detected low concentrations of cf*BRAF^{V600E}* (12 copies/mL), and in the other seven any copy of cf*BRAF^{V600E}* was detected, but only wild type sequences.

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/mL of cf*BRAF*^{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 three patients that harbored the *BRAF*^{V600E} mutation in the tumor tissue, as determined by the Sanger method (data not shown). These three patients, at the moment of the cf*BRAF*^{V600E} determination, had a low tumor burden (40, 38 and 16 mm, respectively). Also, the fractional abundance of cf*BRAF*^{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/mL and the tumor burden ($r=0.742$; $p<0.001$) (Figure 2A). We also found a correlation between the concentrations of cf*BRAF*^{V600E} and the tumor markers MIA ($r=0.708$; $p=0.001$), S100 ($r=0.543$; $p=0.02$), and the enzyme activity of LDH ($r=0.617$; $p=0.007$) (Figure 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, while 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 cf*BRAF*^{V600E} in plasma decreased significantly (median: 0 copies/mL; Q1-Q3: 0-49 copies/mL; $p<0.01$ related to basal) (Figure 3A). The decrease was maintained at the moment of the best response (median: 0 copies/mL; Q1-Q3: 0-33 copies/mL; $p<0.01$ related to basal), but 40% of these patients had detectable cf*BRAF*^{V600E} mutations in blood at that moment. Interestingly, four out of five patients with complete response were negative for circulating cf*BRAF*^{V600E} mutation.

Sixteen plasma samples from patients with progression were available for quantification. Twelve patients (75%) had detectable concentrations of circulating cf*BRAF*^{V600E} mutations (median: 115 copies/mL; Q1-Q3: 3-707 copies/mL; p=0.013 related with best response) (Figure 3A). Furthermore, 6 of these patients had undetectable concentrations of cf*BRAF*^{V600E} mutations at the moment of best response but at progression they had detectable circulating DNA with *BRAF*^{V600E} mutations (Figure 3B and Supplemental Figure 2). During treatment the number of copies/mL 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-years overall survival had lower number of copies/mL of cfBRAF^{V600E} mutations than patients with <2 years overall survival (median copies/mL in >2-y OS patients: 27; Q1-Q3: 0-76; median copies/mL in patients <2-y OS: 478; Q1-Q3: 138-9537; p=0.01) (Figure 4A). Furthermore, considering 216 cfDNA mutant copies/mL as the cut-off, those patients with lower number of basal cf*BRAF*^{V600E} copies/mL had a mean overall survival of 27.7 months (CI95%: 21-34), longer than those with higher basal cfBRAF^{V600E} copies/mL (mean 8.6 months; CI95%: 4-13; p<0.001) (Figure 4B). Also, these patients had a better progression-free survival (median 9 months; CI95%: 0.2-18) as compared with those with higher basal cfBRAF^{V600E} copies/mL (median 3 months; CI95%: 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/mL (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 detecting mutation in blood is that most cfDNA is wild type and the tumour derived mutant DNA fraction in cfDNA can be less than 0.01% (18, 20). We could detect with reproducible results *BRAF*^{V600E} mutation at a lower dilution (Figure 1), confirming that ddPCR was a reliable method to detect cf*BRAF*^{V600E} mutation in blood and more analytically sensitive than other methods (17), and with a specificity similar to those reported by others (19). We have reported our data in copies/mL (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 using copies/mL could be more appropriate for low abundance of DNA, which occurs in plasma. These 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). Nowadays, the selection of patients with melanoma for iBRAF to receive 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 one 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 cf*BRAF*^{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 cf*BRAF*^{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 situations (30), affecting the diagnostic specificity of the cf*BRAF*^{V600E} analysis.

We observed a relationship between basal cf*BRAF*^{V600E} and tumor burden, which is likely a consequence of DNA released to circulation from tumor cells. Interestingly, a higher concentration of cf*BRAF*^{V600E} was associated with a worse clinical outcome. On the contrary, *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 three tumor markers also correlated with cf*BRAF*^{V600E} at baseline. Additionally, since 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/mL are in the logarithmic scale and are very clear in our series of patients. A decrease of the cf*BRAF*^{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 (Supplemental Figure 2) (32).

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 the cf*BRAF*^{V600E} concentrations could be an analytical sign that the therapy is inefficient. Additionally, simultaneous blood analysis of other mutations different to *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-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 cf*BRAF*^{V600E} analysis by ddPCR of blood from patients with melanoma is useful for identifying patients that could benefit of treatment with iBRAF and to monitor response to treatment.

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Table 1. Patient baseline characteristics. Dab.: dabrafenib; Vem.: vemurafenib

Variable	**Overall (N=20)**	Treatment
Mean age-years (SD)	**50 (12)**	
Gender-no. (%)		
Male	**13 (65)**	3 Dab. and 10 Vem.
Female	**7 (35)**	2 Dab. and 5 Vem.
Stage-no. (%)		
Unresectable IIIc	**1 (5)**	0 Dab. and 1 Vem.
IVa	**5 (25)**	2 Dab. and 3 Vem.
IVb	**1 (5)**	0 Dab. and 1 Vem.
IVc	**13 (65)**	3 Dab. and 10 Vem.
Primary site-no. (%)		
Cutaneous	**16 (80)**	4 Dab. and 12 Vem.
Mucosal	**0 (0)**	0 Dab. and 0 Vem.
Uveal	**0 (0)**	0 Dab. and 0 Vem.
Acral	**2 (10)**	0 Dab. and 2 Vem.
Unknown	**2 (10)**	1 Dab. and 1 Vem.

Figures

Figure 1. Analytical sensitivity of digital droplet PCR determination. Genomic DNA from the HT-29 mutant cell line containing the *BRAF*^{V600E} mutation was serially diluted in a constant background of wild-type human genomic DNA from control leukocytes. Results are represented in term of *BRAF*^{V600E} copies/mL (*A*) or fractional abundance in wild type (*B*). Experiments were repeated four times.

Figure 2. (*A*), Pre-treatment relationship between cf*BRAF*^{V600E} concentrations and the tumor burden. (*B*), Pre-treatment relationship between cf*BRAF*^{V600E} concentrations and MIA, S100, and LDH.

Figure 3. (*A*), Individual changes in cf*BRAF*^{V600E} after one month in patients receiving iBRAF therapy (first visit, FV). (*B*), Concentrations of cf*BRAF*^{V600E} in patients at pre-treatment (Basal), at first visit (FV), at the moment of best response (BR) and at the moment of progressive disease (PD). Lines represent the median. (*C*), Evolution of cfBRAF^{V600E} in 6 patients with undetectable concentrations during treatment response but increased at progression.

Figure 4. (*A*), Concentrations of basal cf*BRAF*^{V600E} in patients with ≥2-year OS or <2-year OS. (*B*), Kaplan-Meier plot representing overall survival (left) and progression free survival (right) probabilities for patients with advanced melanoma according to basal cfBRAF^{V600E}. Cut-off for cf*BRAF*^{V600E} concentrations = 216 copies/mL.