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Liquid Profiling of Circulating Tumor DNA in Plasma of Melanoma Patients for Companion Diagnostics and Monitoring of BRAF Inhibitor Therapy

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BACKGROUND: The current standard for determining eligibility of patients with metastatic melanoma for BRAF-targeted therapy is tissue-based testing of *BRAF* mutations. As patients are rarely rebiopsied, detection in blood might be advantageous by enabling a comprehensive assessment of tumor mutational status in real time and thereby representing a noninvasive biomarker for monitoring BRAF therapy.

METHODS: In all, 634 stage I to IV melanoma patients were enrolled at 2 centers, and 1406 plasma samples were prospectively collected. Patients were assigned to 3 separate study cohorts: study 1 for assessment of circulating tumor DNA (ctDNA) as part of companion diagnostics, study 2 for assessment of ctDNA for patients with low tumor burden and for follow-up, and study 3 for monitoring of resistance to BRAF inhibitor (BRAFi) or mitogen-activated protein kinase inhibitor therapy.

RESULTS: Overall, a high degree of concordance between plasma and tissue testing results was observed at 90.9% (study 1) and 90.1% (study 2), respectively. Interestingly, discrepant results were in some cases associated with nonresponse to BRAFi (n = 3) or a secondary *BRAF*-mutant malignancy (n = 5). Importantly, ctDNA results correlated with the clinical course of disease in 95.7% and with response to treatment. Significantly, the detection of *BRAF* mutant ctDNA preceded relapse assessed by Response Evaluation Criteria in Solid Tumors, and was more specific than serum S100 and lactate dehydrogenase.

CONCLUSIONS: Blood-based testing compares favorably with standard-of-care tissue-based *BRAF* mutation testing. Importantly, blood-based *BRAF* testing correlates with the clinical course, even for early-stage patients, and may be used to predict response to treatment, recurrence, and resistance before radioimaging under BRAFi therapy, thereby enabling considerable improvements in patient treatment.

The analysis of tumor-associated genetic alterations in tumor-derived cell-free DNA (cfDNA)[7], commonly referred to as circulating tumor DNA (ctDNA) *(1)*, represents an area of active research. In the past 5 years, a paradigm shift in the treatment of cancer patients has introduced a customized patient-specific approach with layering of numerous therapeutic interventions, including immunotherapy and molecularly targeted agents *(2)*. In case of metastatic malignant melanoma, in which 40% to 50% of patients harbor a *BRAF*[8] V600 mutation *(3)* and are thereby eligible to receive therapy with inhibitors that target *BRAF* mutations (BRAFi) or mitogen-activated protein kinase signaling (MEKi), significant improvements in progression-free survival and overall survival have been achieved *(4–6)*. Unfortunately, most patients inevitably develop resistance to targeted therapy within 6 to 12 months *(4, 7)*.

A requirement for administration of BRAFi/MEKi is the identification of a *BRAF* mutation that is routinely determined by direct sequencing of melanoma tissue

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[7] Nonstandard abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; BRAFi, *BRAF* inhibitor; MEKi, mitogen-activated protein kinase inhibitor; LDH, lactate dehydrogenase; RECIST, Response Evaluation Criteria in Solid Tumors; GE, genome equivalent; BEAMing, beads, emulsification, amplification, and magnetics; FFPE, formalin-fixed, paraffin-embedded; PD, progressive disease; IQR, interquartile range.

[8] Human Genes: *BRAF,* v-raf murine sarcoma viral oncogene homolog B1; *LINE-1,* long interspersed nuclear element-1; *PIK3CA,* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

samples, which may not represent the current somatic mutation status or tumor heterogeneity. This limitation can be overcome by the analysis of ctDNA allowing for real-time comprehensive mutation assessment of all tumor sites within a patient. Beyond the determination of baseline mutational status, the minimally invasive nature of ctDNA sample acquisition enables routine monitoring of response and resistance to targeted therapy. This is of particular importance for melanoma patients because of the lack of clinically useful biomarkers *(8)*.

Despite the inherent advantages of liquid profiling, certain characteristics of ctDNA should be considered to obtain reliable results. Chiefly, its highly fragmented nature *(9–11)* and the minor fraction of ctDNA with a variable contribution of 0.01 to >10% of total circulating DNA *(11, 12)* are of high importance with respect to the desired sensitivity of the selected detection method and the preanalytical sample handling.

Here, we report on the results of a large-scale translational melanoma study with the primary aim of investigating the practicality of ctDNA analysis and its viability to serve as an alternative to tissue-based testing to assess *BRAF* mutation status. Second, we evaluated whether quantitative serial measurements of *BRAF* V600E mutant cfDNA can predict resistance to BRAFi/MEKi therapy.

Materials and Methods

STUDY DESIGN AND PATIENTS

This exploratory, translational, 2-center study recruited melanoma patients at German university medical centers in Mannheim and Essen, and was conducted following approval by an institutional review board. Informed consent was obtained from each participant or the individual's guardian before sample collection and analysis. Between 2011 and 2015, 634 melanoma patients were enrolled, and 1406 plasma samples were prospectively collected. Patient enrollment and study overview are presented in Fig. 1. Patients were assigned to 3 study cohorts: (*a*) a concordance study of stage III and IV melanoma patients for assessment of ctDNA suitability as part of companion diagnostics (study 1), (*b*) a concordance and observational study including all stages of disease for assessment of ctDNA suitability for patients having a low tumor burden and monitoring of patients over time (study 2), and (*c*) an observational study of patients undergoing BRAFi/MEKi therapy to evaluate the suitability of ctDNA for monitoring resistance to targeted therapy (study 3).

All patients were treated according to standard-of-care guidelines or as part of clinical trials approved by our institutional review boards. Collection of blood samples for assessment of ctDNA, serum markers lactate dehydrogenase (LDH) and S100, and radioimaging diagnostics were performed as clinically indicated. Imaging studies were reviewed according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 *(13)*, and tumor load was determined by the sum of diameters of all measurable lesions. For all patients, mutational data from archival tumor specimens were available.

PROCEDURES

K_3-EDTA-blood samples were centrifuged at 1600*g* for 10 min at 4 °C within 6 h after collection with few exceptions. Supernatant was transferred to fresh tubes, pooled, centrifuged at 3000*g* for 10 min at ambient temperature, and stored at −80 °C. cfDNA was extracted from 2 mL of plasma using QIAamp Circulating Nucleic Acid Kits (Qiagen) and stored at −20 °C.

The total amount of isolated cfDNA was quantified using a previously described version of a 79-bp human *LINE-1* quantitative real-time PCR *(9, 14, 15)*. Amplification was performed on a Step One Plus™ real-time PCR system (Thermo Fisher Scientific) using the Platinum® SYBR Green quantitative PCR SuperMix-UDG (Thermo Fisher Scientific) in a 15-μL reaction consisting of 3 μL of template DNA according to the manufacturer's instructions. Cycling conditions were 95 °C for 2 min, then 95 °C for 15 s and 60 °C for 30 s cycled 40 times. Intact human genomic DNA (Promega) serially diluted from 303 to 0.3 genome equivalent (GE)/μL (1 GE corresponding to 3.3 pg) was used as a reference standard. Each sample was run in duplicate; each reference, in triplicate.

Plasma DNA was analyzed for *BRAF* V600E mutation with BEAMing (beads, emulsification, amplification, and magnetics) *(16)* using the OncoBEAM™ V600E assay (Sysmex Inostics) according to the manufacturer′s instructions. All experiments were conducted at the University Medical Centre Mannheim. Briefly, as described previously *(15, 16)*, the total amount of cfDNA purified from 2 mL of plasma (140 μL minus the volume used for the *LINE-1* quantitative PCR assay) was used in 6 multiples of 65-μL reactions in the initial target-specific V600E spanning PCR. PCR products were pooled and quantified using the Qubit™ dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). For each sample, 5 μL of PCR product, diluted in low EDTA-Tris EDTA (TE) buffer, pH 8.0, to reach a concentration of 20 pmol/L, was used for emulsion PCR. After breaking the emulsion PCR reaction, wild-type and mutant-specific probes were hybridized, and flow cytometry analysis was conducted using the BD Accuri™ C6 Cytometer (BD Bioscience). For an assay to be scored as positive, the mutant fraction had to exceed the prespecified cutoff (0.03%), the absolute number of mutant beads had to be >5, and polyclonal beads had to be detected according to their Poisson distribution.

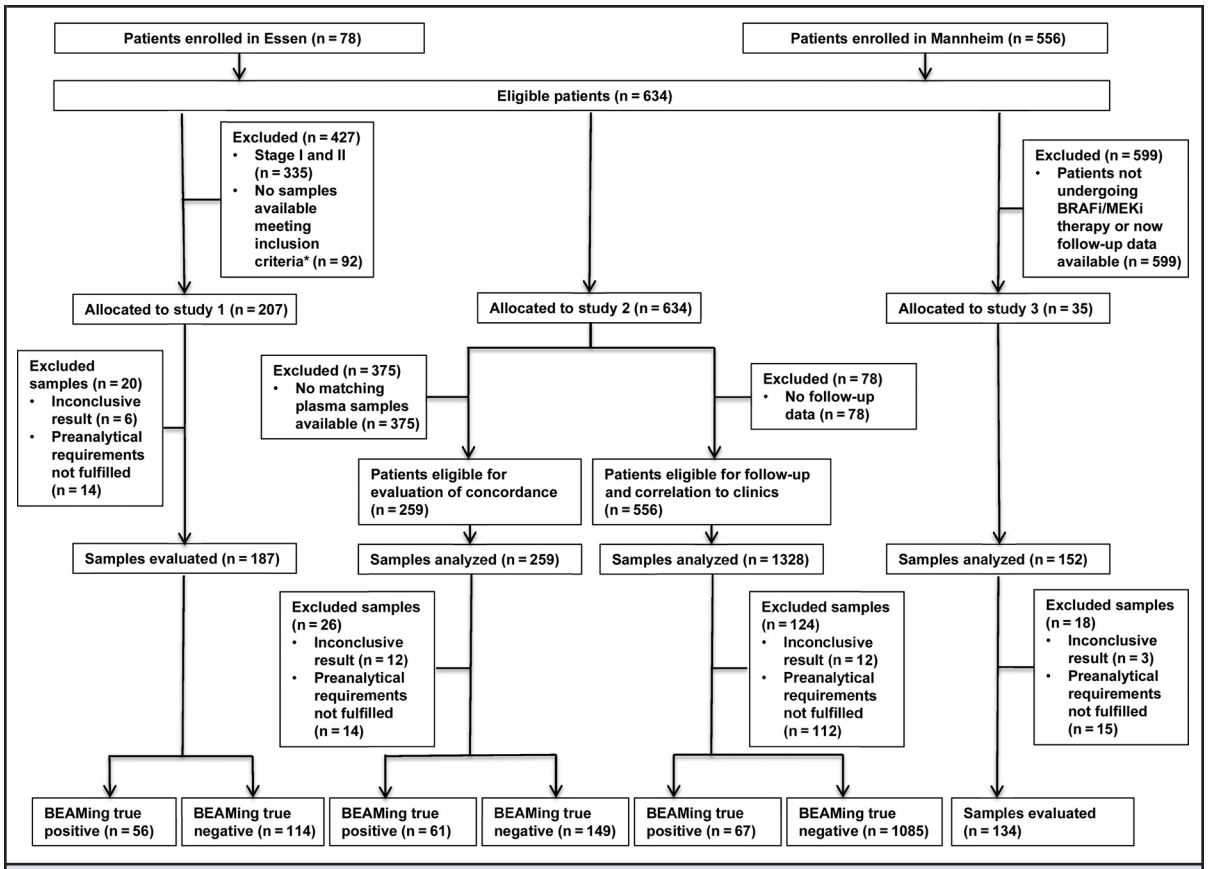

Fig. 1. Patient enrollment and sample collection.

The flow diagram displays the study design. A total of 634 patients were enrolled between 2011 and 2015 at 2 centers. These patients were assigned to 3 separate studies: 207 patients for concordance study 1; 634 patients for study 2, with 259 patients considered for assessment of concordance and 556 patients evaluated for follow-up; and 35 patients for monitoring of BRAFi/MEKi therapy in study 3. * Inclusion criteria for assessment of concordance between testing modalities was a blood draw performed at a time point when patients were either treatment-naïve or receiving treatment after showing PD according to RECIST, version 1.1.

Mutational analysis of tumor tissue DNA was performed from archived formalin-fixed, paraffin-embedded (FFPE) tumor specimens as part of standard care. The mutational status was determined using Sanger sequencing in a routine setup.

Investigators performing mutation analysis of plasma DNA with BEAMing were blinded to the results of mutation analysis of tumor tissue.

STATISTICAL ANALYSIS

Statistical analysis to determine the agreement between *BRAF* mutation status in ctDNA and tumor tissue was assessed by calculation of positive, negative, and overall percentage agreement. In addition, concordance was calculated using the κ coefficient.

Subsequently, we compared the results of ctDNA, S100, and LDH assays and the results of imaging studies to assess their ability to detect progressive disease (PD) under BRAFi therapy. PD was defined based on radiographic and clinical findings. Radiographic disease progression included PD or death. A Wilcoxon signed rank test was used to calculate the *P* value for evaluating the difference in lead time between ctDNA and S100, ctDNA vs LDH, and ctDNA vs imaging studies.

For all statistical analyses, *P* values <0.05 were considered statistically significant. All statistical analyses were carried out using GraphPad Software and R version 3.0.1 (The R Foundation for Statistical Computing).

Results

For this translational study, 1406 samples of 634 melanoma patients were collected prospectively, patients were assigned to 3 separate study cohorts as described above, and samples were analyzed by BEAMing.

Preexperimental analytical validation studies were performed to determine assay sensitivity. Detailed information is provided in Fig. 1 and Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue5.

STUDY 1: SUITABILITY OF ctDNA FOR ASSESSMENT OF *BRAF* V600E MUTATIONAL STATUS AS PART OF COMPANION DIAGNOSTICS

In the first study, 207 stage III and IV melanoma patients were enrolled. Only patients with blood samples available and either temporally matched to archival tumor tissue or obtained when patients showed PD according to RECIST 1.1 were included. Patients receiving treatment with BRAFi/MEKi and sample collection exclusively performed when showing measurable responses were excluded (Fig. 1). Clinical characteristics are listed in Table 1; detailed clinical information is provided in Table 2 of the online Data Supplement.

All patients were tested for *BRAF* V600E mutation in FFPE tumor samples by Sanger sequencing, and cfDNA samples were analyzed by BEAMing (Table 1). Twenty samples were excluded from evaluation because either the preanalytical requirements for sample handling were not fulfilled or the results obtained by BEAMing were inconclusive. The frequency of individual *BRAF* mutations in plasma DNA was generally consistent with data reported in the Catalogue of Somatic Mutations in Cancer database *(17)*. Examples of typical plasma assay results from representative individuals are shown in Fig. 2 of the online Data Supplement. Compared with cfDNA levels, ctDNA levels reflected the tumor stage more precisely. Comparison of the testing modalities revealed a high degree of concordance, with an overall agreement between plasma and tumor tissue testing in 90.9% (Table 1). In 2 cases of false-positive results, retesting of tumor tissue revealed a *BRAF* V600E mutation. Therefore, these cases were considered as concordant and highlight the variability in current standard-of-care tissue techniques. For all other tissue/blood discordances and samples collected in Essen (n = 5), plasma and archival tumor tissue results were confirmed by repeating BEAMing analysis at Sysmex Inostics. As there was insufficient material available, retesting could not be performed for the discordant results of the samples obtained in Mannheim (n = 12). Interestingly, for 2 of those cases, a secondary *BRAF* V600E-positive malignancy was found to be responsible for the discrepant results (2 of 7 false-positive results). Furthermore, 3 of 5 patients with negative results in plasma, but with positive tests in archival tumor tissue, did not respond to BRAFi therapy potentially because of a false-positive tissue test result. In the remaining discordant cases in which a *BRAF* V600E mutation was not detected in plasma, but was detected in tissue, the discrepancy may be attributable to instances in which ctDNA was not shed into the circulation or the quantity was not sufficient for detection. In particular, patients with concordant results between testing modalities had a higher mean ctDNA fraction [mean, 1.325; median, 0.007; interquartile range (IQR), 0.036] compared with a mean ctDNA fraction at the detection limit of BEAMing for the subgroup of patients with discordant results (mean, 0.085; median, 0.015; IQR, 0.032).

STUDY 2: SUITABILITY OF ctDNA FOR ASSESSMENT OF *BRAF* V600E MUTATIONAL STATUS FOR EARLY STAGES OF DISEASE AND FOR FOLLOW-UP OF PATIENTS

In the second study, 634 stage I to IV melanoma patients were included and separated into 2 subgroups: (*a*) evaluation of concordance between testing modalities and (*b*) evaluation of ctDNA suitability for follow-up of patients.

First, to evaluate whether ctDNA analysis is also suitable for assessment of *BRAF* V600E tumor mutational status of patients with low tumor burden, all patients with blood samples available, either temporally matched to archival tumor tissue or obtained when patients showed PD according to RECIST 1.1, were evaluated in respect to the concordance between plasma and tumor-tissue testing. In total, 259 patients met these inclusion criteria. Clinical characteristics and results for tissue and plasma-based mutation testing are shown in Table 2, and detailed clinical information is provided in Table 2 of the online Data Supplement. Similar to the results of study 1, the comparison of plasma and tissue testing revealed a high overall agreement of 90.1% (Table 2). As expected, the overall agreement slightly decreased for earlier stages of disease and ranged from 85.2% to 93.2%. Interestingly, the number of false-positive results increased, whereas the number of false-negative results decreased for earlier stages of disease (Table 2). Considering the increasing deviation of the mutation frequency from that reported in the Catalogue of Somatic Mutations in Cancer database for the results of tissue-based testing (stages IV to I: 35.3%, 27.8%, 16.7%, 4.5%) and the more reliable frequency obtained by BEAMing (stages IV to I: 36.1%, 35.2%, 29.2%, 22.2%), the increasing percentage of false-positive results could most likely be explained by an underestimation of *BRAF* V600E mutation by tissue-based analysis.

Second, for assessment of ctDNA suitability to monitor patients over time, all patients with follow-up data available (clinical evaluation, serum biomarkers, radioimaging) were evaluated. In total, 556 patients met these eligibility criteria, and 1328 samples were available for cfDNA analysis, with an average of 2.8 tests being evaluated per patient (Fig. 1). Clinical

Table 1. Patient characteristics and results of study 1.

Number of patients considered for evaluation	Study 1[a] Patients recruited in Mannheim n = 116 (%)	Patients recruited in Essen n = 71 (%)	Cumulative n = 187 (%)
Stage			
IV	62 (53.5)	71 (100)	133 (71.1)
III	54 (46.5)	0 (0)	54 (28.9)
Tissue-based analysis			
BRAF V600E mutation	37 (31.8)	25 (35.2)	62 (33.2)
Other *BRAF* mutation	4 (3.5)	9 (12.7)	13 (7.0)
NRAS mutation	11 (9.5)	12 (16.9)	23 (12.3)
cKIT mutation	4 (3.5)	NA[b]	4 (2.1)
No mutation	60 (51.7)	25 (35.2)	85 (45.5)
NA	0 (0)	0 (0)	0 (0)
Plasma-based analysis			
Number of samples analyzed	116	71	187
Mean number of tests per patient	1.0	1.0	1.0
BRAF V600E mutation	39 (33.6)	25 (35.2)	64 (34.2)
No mutation	77 (66.4)	46 (64.8)	123 (65.8)
Therapy			
Treatment-naïve	88 (75.6)	56 (78.9)	144 (77.0)
On treatment	28 (24.4)	15 (21.1)	43 (23.0)
NA	0 (0)	0 (0)	0 (0)
Plasma-based analysis			
Positive for *BRAF* V600E	39 (33.6)	25 (35.2)	64 (34.2)
Negative for *BRAF* V600E	77 (66.4)	46 (64.8)	123 (65.8)
False negative[c]	5 (4.3)	4 (5.6)	9 (4.8)
False positive[d]	7 (6.0)	1 (1.4)	8 (4.3)
Statistical analysis			
Positive agreement	32/37 (86.5)	24/25 (96.0)	56/62 (90.3)
Negative agreement	72/79 (91.1)	42/46 (91.3)	114/125 (91.2)
Overall agreement	104/116 (89.7)	66/71 (92.9)	170/187 (90.9)
κ	0.765	0.85	0.799
SE of κ	0.064	0.064	0.046
95% CI	0.64–0.89	0.72–0.98	0.71–0.89
Number of agreements expected by chance	64.9 (55.9)	37.7 (53.1)	102.5 (54.8)
cfDNA level (GE/mL): mean/median/IQR[e]			
IV	110 000/57 000/61 000	96 000/58 000/48 000	102 000/57 000/53 000
III	56 000/49 000/29 000	NA	56 000/49 000/29 000
III–IV	NA	NA	89 000/54 000/49 000

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Table 1. Patient characteristics and results of study 1. *(Continued from page 834)*

Number of patients considered for evaluation	Study 1[a] Patients recruited in Mannheim n = 116 (%)	Patients recruited in Essen n = 71 (%)	Cumulative n = 187 (%)
ctDNA level, GE/mL: mean/median/IQR			
IV	1393/3/24	7342/0/70	4569/3/48
III	13/3/9	NA	13/3/9
III–IV	NA	NA	3253/3/24
ctDNA fraction, %: mean/median/IQR			
IV	1.17/0.01/0.03	2.13/0.01/0.16	2.13/0.01/0.16
III	0.02/0.01/0.03	NA	0.02/0.01/0.03
III–IV	NA	NA	1.21/0.01/0.03

[a] Table entries displayed as number (percentage) except as noted.
[b] NA, not applicable.
[c] Wild type instead of *BRAF* V600E mutant.
[d] *BRAF* V600E mutant instead of wild type.

characteristics are listed in Table 3; detailed clinical information is provided in Table 3 of the online Data Supplement. The results of ctDNA analysis were compared with the current clinical situation of the patient assessed by the treating oncologist (based on tumor tissue mutational status, radioimaging results, physical examination, blood biomarkers), and an analysis of concordance for each time point was conducted to serve as surrogate for correctness of ctDNA results. This analysis revealed a high level of concordance of 95.7%, ranging from 91.7% to 98.4% depending on tumor stage. Comparable with study 1, in 5 cases a secondary *BRAF* V600E-positive malignancy was found to be responsible for false-positive results. Moreover, in 3 patients with no visible tumor load but undergoing an excision of benign nevus at the time of blood draw, a *BRAF* V600E mutation was detected in plasma at this time.

STUDY 3: SUITABILITY OF ctDNA FOR MONITORING THE COURSE OF BRAFi/MEKi THERAPY

To evaluate the clinical value of measuring the baseline level of circulating *BRAF*-mutant cfDNA with respect to predicting response to targeted therapy and subsequent utility as a monitoring tool, 35 stage IV melanoma patients were prospectively enrolled in study 3. On average, 3.8 samples were analyzed per patient with a median follow-up time of 14.3 months, defined as the time between first presentation during the surveillance period and either 90 days after the last assessment of cfDNA or death. Basic clinical characteristics are provided in Table 4, and detailed information is available in Table 4 of the online Data Supplement.

When comparing the performance of ctDNA with the performance of imaging techniques, fluctuations in ctDNA are generally correlated with treatment response seen on imaging. For all patients responding to therapy, ctDNA levels were below the cutoff after administration of BRAFi/MEKi therapy. During the follow-up period, a PD was detected in 18 cases by radioimaging; ctDNA levels rebounded in 11 cases before radioimaging (by a mean of 177 days), in 3 cases at the time of imaging, and in 1 after progression was noted on imaging (see Fig. 3 in the online Data Supplement). In 3 cases, no increase of ctDNA level was detected at the time of PD as assessed by computed tomography. Interestingly, 2 of these patients showed PD exclusively in the brain, with an observed increase in ctDNA, although remaining below the cutoff. This is most likely attributable to the blood–brain barrier inhibiting the release of ctDNA into peripheral circulation. The course of 3 exemplary patients is shown in Fig. 2 here and in Fig. 4 of the online Data Supplement.

In general, a PD reflected by an increase in *BRAF* V600E mutant cfDNA level was detected substantially earlier than PD assessed by imaging techniques, with a mean lead time reduction of 110 days (SD, 190.7). Compared with biomarker S100 and LDH, there was no significant difference. However, in comparing the baseline with the first response, *BRAF* V600E mutant cfDNA fraction decreased significantly ($P < 0.001$), providing a significant advantage in response detection compared

Table 2. Patient characteristics and results of study 2–evaluation of concordance.

Number of patients considered for evaluation	Study 2–evaluation of concordance[a]				
	Stage IV n = 133 (%)	Stage III n = 54 (%)	Stage II n = 24 (%)	Stage I n = 22 (%)	Cumulative n = 233 (%)
Stage					
IV	133 (100)	NA[b]	NA	NA	133 (57.1)
III	NA	54 (100)	NA	NA	54 (23.2)
II	NA	NA	24 (100)	NA	24 (10.3)
I	NA	NA	NA	21 (100)	21 (9.0)
Tissue-based analysis					
BRAF V600E mutation	47 (35.3)	15 (27.8)	4 (16.7)	1 (4.5)	67 (28.8)
Other *BRAF* mutation	4 (3.0)	0 (0)	1 (4.2)	0 (0)	5 (2.1)
NRAS mutation	7 (5.3)	4 (7.4)	2 (8.3)	0 (0)	13 (5.6)
cKIT mutation	2 (1.5)	2 (3.7)	0 (0)	0 (0)	4 (1.7)
No mutation	73 (54.9)	33 (61.1)	17 (70.8)	21 (95.5)	144 (61.8)
NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Plasma-based analysis					
Number of samples analyzed	133	54	24	21	233
Mean number of tests per patient	1.0	1.0	1.0	1.0	1.0
BRAF V600E mutation	48 (36.1)	19 (35.2)	7 (29.2)	4 (22.2)	78 (33.5)
No mutation	85 (63.9)	35 (64.8)	17 (70.8)	18 (85.7)	155 (66.5)
Plasma-based analysis					
Positive for *BRAF* V600E	48 (36.1)	19 (35.2)	7 (29.2)	4 (22.2)	78 (33.5)
Negative for *BRAF* V600E	85 (63.9)	35 (64.8)	17 (70.8)	18 (85.7)	155 (66.5)
False negative[c]	4 (3.0)	2 (3.7)	0 (0)	0 (0)	6 (2.6)
False positive[d]	5 (3.8)	6 (11.1)	3 (12.5)	3 (13.6)	17 (7.3)
Statistical analysis					
Positive agreement	43/47 (91.5)	13/15 (86.7)	(4/4/100)	1/1 (100)	61/67 (91.0)
Negative agreement	81/86 (94.2)	33/39 (84.6)	17/20 (85.0)	18/21 (85.7)	149/166 (89.8)
Overall agreement	124/133 (93.2)	46/54 (85.2)	21/24 (87.5)	19 (86.4)	210/233 (90.1)
κ	0.853	0.659	0.654	0.353	0.77
SE of κ	0.047	0.109	0.175	0.265	0.045
95% CI	0.760–0.946	0.445–0.872	0.310–0.998	(−) 0.166–0.872	0.682–0.859
Number of agreements expected by chance	71.9 (54.1)	30.6 (56.6)	15.3 (63.9)	17.4 (78.9)	132.9 (57.02)

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Table 2. Patient characteristics and results of study 2–evaluation of concordance. *(Continued from page 836)*

Number of patients considered for evaluation	Study 2–evaluation of concordance[a]				
	Stage IV n = 133 (%)	Stage III n = 54 (%)	Stage II n = 24 (%)	Stage I n = 22 (%)	Cumulative n = 233 (%)
cfDNA level, GE/mL: mean/median/IQR	102 000/57 000/53 000	56 000/50 000/29 000	59 000/52 000/34 000	58 000/42 000/34 000	83 000/53 000/42 000
ctDNA level, GE/mL: mean/median/IQR	4568/3/48	13/3/9	11/7/12	13/6/10	2613/3/19
ctDNA fraction, %: mean/median/IQR	0.02/0.02/0.02	0.02/0.01/0.03	0.02/0.02/0.02	0.02/0.02/0.02	0.02/0.02/0.02

[a] Table entries displayed as number (percentage) except as noted.
[b] NA, not applicable.
[c] Wild type instead of *BRAF* V600E mutant.
[d] *BRAF* V600E mutant instead of wild type.

with serum S100 ($P < 0.04$) or serum LDH levels (not significant).

Discussion

Our analysis reveals a high degree of concordance of *BRAF* V600E mutation status between standard-of-care analysis of archival tumor tissue and plasma-based testing of cfDNA with an overall agreement ranging from 92.3% to 94.5% in the 3 study subpopulations.

Thus, our results support the routine use of ctDNA analysis to establish tumor genotype at diagnosis when treatment with targeted therapies is considered. Additionally, ctDNA provided reliable results for determining tumor genotype in early-stage melanoma patients, in whom ctDNA levels are often found to be very low *(18, 19)*.

To the best of the authors' knowledge, this is the largest study published so far including all melanoma stages and an assessment of *BRAF* V600E mutation status in circulating DNA at serial time points for patient monitoring. This investigation provides substantial evidence that ctDNA analysis is suitable for earlier stage patients with very low ctDNA levels, although the level of concordance slightly decreased. Interestingly, the *BRAF* V600E mutation frequency in earlier stages of disease decreased to a higher extent if determined by tissue-based testing than for ctDNA analysis. This highlights that tissue-based testing of small tumors with a low proportion of tumor cells might be prone to error, and plasma-based testing might reflect tumor mutational status more precisely, even or especially for earlier stages of disease. Moreover, the results of the observational study demonstrate that consistent results can be achieved over time with strong correlation to a patient's clinical course. Although most of the results were consistent with the clinical course of disease, the development of a secondary *BRAF* V600E-positive malignancy was determined to be responsible for the detection of *BRAF* V600E mutant fragments in patients whose tumor tissue was *BRAF* wild-type, thus confounding interpretation. Furthermore, in ctDNA *BRAF* wild-type patients, a 1-time positivity in circulation was found to be directly correlated to an excision of common benign melanocytic nevi, known to harbor *BRAF* mutations with a high frequency *(20)*, that might have shed *BRAF*-mutant fragments into the circulation by disruption. This observation is in agreement with a previously described case report *(21)* and the findings of increased ctDNA levels after surgical interventions *(20, 22)*. Thus, a single time point positivity might not represent recurrence of disease and warrants further evaluation.

A remarkable strength of our study is the use of prospectively collected blood samples facilitating a state-of-the-art diagnosis adhering to all applicable

Table 3. Patient characteristics and results of study 2–follow-up of patients.

Number of patients considered for evaluation	Study 2–follow-up[a]				
	Stage IV n = 64 (%)	Stage III n = 177 (%)	Stage II n = 85 (%)	Stage I n = 167 (%)	Cumulative n = 433 (%)
Stage					
IV	64 (100)	NA[b]	NA	NA	64 (14.8)
III	NA	117 (100)	NA	NA	117 (27.0)
II	NA	NA	85 (100)	NA	85 (19.6)
I	NA	NA	NA	167 (100)	167 (38.6)
Tissue-based analysis					
BRAF V600E mutation	31 (48.4)	31 (26.5)	7 (8.2)	13 (7.8)	82 (18.9)
Other *BRAF* mutation	3 (4.7)	0 (0)	1 (1.2)	0 (0)	4 (0.9)
NRAS mutation	5 (7.8)	4 (3.4)	2 (2.4)	0 (0)	11 (2.5)
cKIT mutation	2 (3.1)	2 (1.7)	0 (0)	0 (0)	4 (0.9)
no mutation	23 (35.9)	80 (68.4)	75 (88.2)	154 (92.2)	343 (79.2)
NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Plasma-based analysis					
Number of samples analyzed	204	394	245	361	1204
Mean number of tests per patient	3.2	3.4	2.9	2.2	2.8
BRAF V600E mutation	37 (18.1)	32 (8.1)	8 (3.3)	13 (3.6)	90 (7.5)
No mutation	167 (81.9)	362 (91.9)	237 (96.7)	348 (96.4)	1114 (92.5)
Plasma-based analysis					
Positive for *BRAF* V600E	37 (18.1)	32 (8.1)	8 (3.3)	13 (3.6)	90 (7.5)
Negative for *BRAF* V600E	167 (81.9)	349 (91.9)	237 (96.7)	348 (96.4)	1114 (92.5)
False negative[c]	15 (7.4)	13 (3.3)	1 (0.4)	0 (0)	29 (2.4)
False positive[d]	2 (0.9)	8 (2.0)	3 (1.2)	10 (2.8)	23 (1.9)
Statistical analysis					
Positive agreement	35/50 (70.0)	24/37 (64.9)	5/6 (83.3)	3/3 (100)	67/96 (69.8)
Negative agreement	152/154 (98.7)	349/357 (97.8)	236/239 (98.7)	348/358 (97.2)	1085/1108 (97.9)
Overall agreement	187/204 (91.7)	373/394 (94.7)	241/245 (98.4)	351/361 (97.2)	1152/1204 (95.7)
κ	0.75	0.667	0.706	0.366	0.697
SE of κ	0.056	0.068	0.14	0.153	0.04
95% CI	0.643–0.863	0.534–0.800	0.432–0.980	0.067–0.666	0.619–0.775
Number of agreements expected by chance	135.1 (66.2)	331 (84.0)	231.4 (94.5)	345.2 (95.6)	1032 (85.7)

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Table 3. Patient characteristics and results of study 2–follow-up of patients. *(Continued from page 838)*

	Study 2–follow-up[a]				
Number of patients considered for evaluation	Stage IV n = 64 (%)	Stage III n = 177 (%)	Stage II n = 85 (%)	Stage I n = 167 (%)	Cumulative n = 433 (%)
cfDNA level, GE/mL: mean/median/IQR	111 000/58 000/53 000	55 000/47 000/31 000	55 000/47 000/33 000	56 000/44 000/37 000	64 000/48 000/38 000
ctDNA level, GE/mL: mean/median/IQR	1510/7/32	11/4/4	5/3/4	11/7/11	232/4/6
ctDNA fraction, %: mean/median/IQR	0.69/0.01/0.04	0.02/0.01/0.01	0.01/0.01/0.01	0.01/0.01/0.01	0.12/0.01/0.01

[a] Table entries displayed as number (percentage) except as noted.
[b] NA, not applicable.
[c] Wild type instead of *BRAF* V600E mutant.
[d] *BRAF* V600E mutant instead of wild type.

Table 4. Patient characteristics BRAFi/MEKi study.

Number of patients	BRAFi cohort n = 35 (%)
Stage IIIC (unresectable)	4 (11.4)
Stage IV	31 (88.6)
M1a	1 (2.9)
M1b	4 (11.4)
M1c	26 (74.3)
LDH ↑	20 (57.1)
Brain metastases	18 (51.4)
BRAF status	
V600E	35 (100)
BEAMing positive	35 (100)
Plasma-based analysis	
Number of samples analyzed	134
Mean number of tests per patient	3.83
cfDNA level, GE/mL: mean/median/IQR	96 000/59 000/46 000
ctDNA level, GE/mL: mean/median/IQR	2789/13/282
ctDNA fraction, %: mean/median/IQR	1.27/0.02/0.36
BRAFi/MEKi treatment	
V+C	7 (20.0)
D+T	10 (28.6)
V mono	15 (42.9)
D mono	3 (8.6)
Median duration of treatment, months	17.7
Median time to resistance, months	9.1
Median overall survival (95% CI), months	23.0 (14.6-27.9)
Line of treatment	
First-line	32 (91.4)
Second-line	2 (5.7)
Third-line	1 (2.9)
Best overall response	
Complete response	4 (11.4)
Partial response	11 (31.4)
Stable disease	9 (25.8)
Progressive disease	11 (31.4)

recommendations, recently published as a technical rule by DIN CEN *(23)*. Previous studies have demonstrated that detection of *BRAF* V600E in melanoma patients is feasible in plasma; nevertheless, the level of concordance between tissue- and plasma-based testing varies widely, ranging from 56% to 91% *(24–28)*. Those studies with a high degree of concordance were limited by sample size *(25, 27)*. However, the vast majority of studies reported a level of concordance <76% *(19)*. Compared with the present study and other studies with a high level of concordance either for melanoma *(25, 27)* or other malignancies *(12, 29, 30)*, preexamination procedures varied widely. Varying plasma volumes *(26)*, serum being used for cfDNA extraction instead of plasma *(24, 31)*, vari-

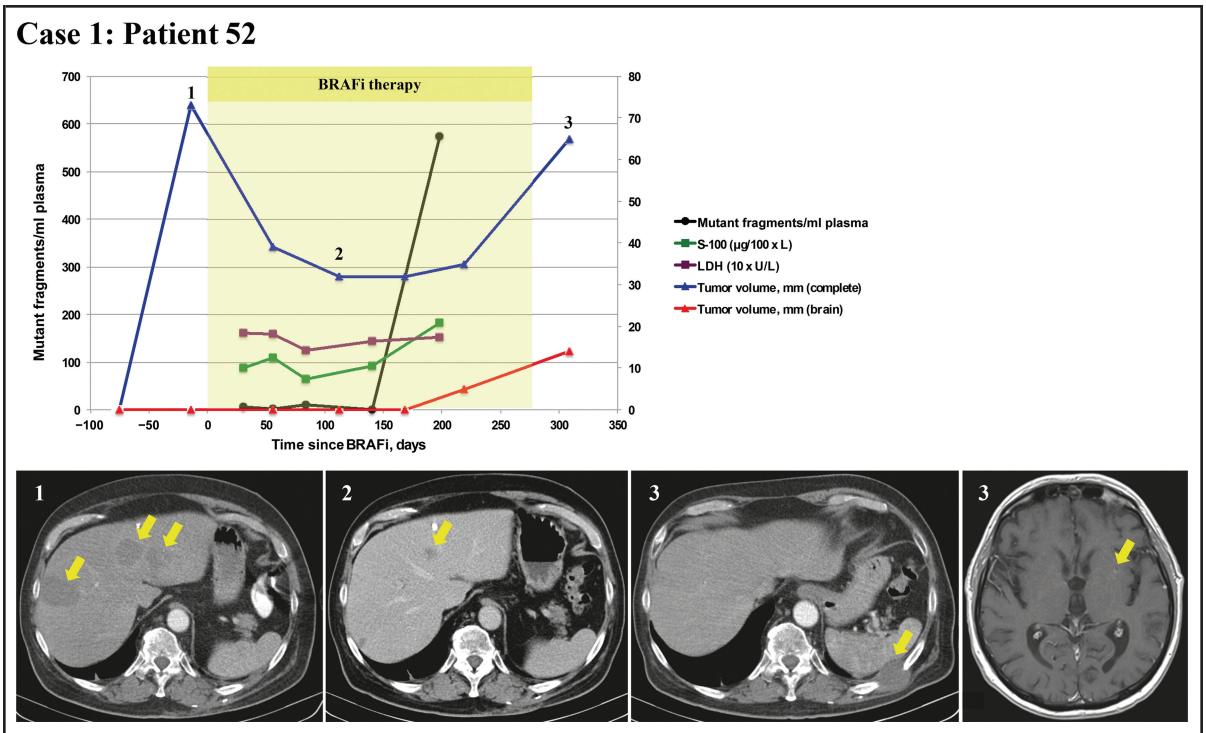

Fig. 2. *BRAF* **V600E mutant ctDNA monitoring during clinical disease course.**

Serial monitoring of ctDNA in 1 melanoma patient undergoing BRAFi/MEKi therapy. Level of mutant *BRAF* V600E fragments (GE/mL plasma) is displayed (black) and compared with total tumor load in millimeters (blue line) and intracranial tumor load in millimeters (red line) as assessed by imaging techniques and determined by the sum of diameters of all measurable lesions. Exemplary computed tomography (CT)/magnetic resonance imaging scans of different time points are displayed. The time of imaging assessments is indicated by numbers. Additionally, the concentration of serum LDH (10 × U/L) (purple line) and serum S100 (µg/100 × L) (green line) during follow-up is shown. Treatment duration of BRAFi/MEKi is highlighted in yellow. To enable plotting of all data sets on the same graph, the left *y* axis plots ctDNA (copies/mL) and the right *y* axis plots LDH, S100, and tumor load. Patient 52 improved clinically with partial response (PR) assessed by CT scans according to RECIST v1.1 and illustrated by a significant regression of liver metastases after initiation of vemurafenib (BRAFi) and cobimetinib (MEKi). ctDNA levels were fluctuating around the cutoff during time of PR and clearly exceeding the cutoff 23 days before PD detected by imaging, which revealed new metastases (e.g., in brain and soft tissue). S100 and LDH paralleled ctDNA level.

able plasma volumes as input for testing *(26)*, and the time between blood draw and plasma separation being disregarded *(28, 31)* may have resulted in leukocyte lysis and subsequent contamination with an overabundance of wild-type DNA, resulting in an appreciable dilution of mutant fragments and subsequent false-negative results. The clinical implication of this was highlighted by Santiago-Walker and colleagues, who detected *BRAF* mutations in cfDNA in 556 of 732 (76%) patients with a *BRAF* mutant-positive tumor and who were receiving either BRAFi or MEKi treatment. Depending on plasma-based results, 24% of patients would not have been eligible for BRAFi/MEKi therapy. Importantly, these patients had a longer progression-free survival and overall survival and showed a higher response rate *(28)*. Thus, 25% of patients would not have benefited from targeted therapies, which is most likely because of a dilution of ctDNA under the detection limit.

In our study, the concordance of *BRAF* V600E plasma testing using BEAMing technology with a reported sensitivity of 0.01% *(9, 18)* and tumor tissue analysis did not reach 100%. This finding is not surprising, given the fact that using archival tumor specimens as a reference for current mutational status presents numerous shortcomings, including the inherent median error rate of 1.44% for genetic tests *(32)* and intratumoral heterogeneity *(12)*. The notion of tumor heterogeneity is consistent with our findings in 2 cases for which a *BRAF* mutation was revealed by retesting of tumor tissue, which is in accordance with the results of earlier studies. Pinzani reported that 20% of initially *BRAF* wild-type tissue samples were positive if reanalyzed with a more sensitive

method *(33)*; Higgins and colleagues improved the concordance between *PIK3CA* mutational status of FFPE specimens of breast cancer patients and temporally matched plasma samples from 73% to 100% by retesting FFPE samples with BEAMing *(29)*.

Currently, only a few studies have examined the clinical utility of ctDNA in melanoma *(22, 27, 34–36)*, most in small patient populations. In agreement with the largest studies published so far *(34, 35)*, this study showed that serial assessment of ctDNA is suitable for monitoring response to targeted therapy, as fluctuations in ctDNA generally correlated with treatment response. In line with previous studies, this study proved that ctDNA generally revealed resistance to therapy earlier than imaging *(26, 34)* and extended these findings by providing statistically significant evidence of lead-time reduction compared with RECIST by a median of 110 days. Moreover, ctDNA analysis demonstrates superior specificity than that of other circulating biomarkers, with statistical significance not only for serum LDH as previously described *(35)* but also for serum S100. Therefore, the emergence of a *BRAF* mutation or an increase in fraction during treatment will provide an early warning of progression and could serve as the rationale to reassess treatment at the earliest possible point allowing for a favorable clinical outcome. To prove the clinical benefit of monitoring melanoma patients by ctDNA analysis, further prospective, randomized clinical studies are warranted.

In summary, our study demonstrates that with a simple single blood draw, *BRAF* V600E mutation status can be determined reliably to assess the suitability for targeted BRAFi/MEKi therapies. Additionally, this study highlights the suitability of ctDNA as a general monitoring tool for a clinical course of melanoma even for early stages, and for responses to targeted therapies. Moreover, the assessment of *BRAF* mutations in circulating DNA significantly improves the detection of resistance to treatment compared with imaging techniques or serum markers such as S100 and LDH and, therefore, could become an integral part of clinical diagnostics for melanoma patients.

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V. Haselmann, J. Utikal, D. Schadendorf, and M. Neumaier designed the study. V. Haselmann, C. Gebhardt, C. Czerwinski, A. Sucker, J. Utikal, and D. Schadendorf were responsible for data collection and management. C. Gebhardt, A. Sucker, J. Utikal, and D. Schadendorf enrolled patients. V. Haselmann, I. Brechtel, and A. Duda established and improved methodology and were responsible for ctDNA isolation and analyses. T. Holland-Letz and V. Haselmann were responsible for biostatistics analyses. V. Haselmann, C. Gebhardt, and A. Duda were responsible for interpretation of data. V. Haselmann and C. Gebhardt prepared the tables and figures. V. Haselmann did drafting of manuscript. All authors contributed to revision of the manuscript and approved it for submission.

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