Prospective Blinded Study of BRAF<sup>V600E</sup> Mutation Detection in Cell-Free DNA of Patients with Systemic Histiocytic Disorders

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ABSTRACT

Patients with Langerhans cell histiocytosis (LCH) and Erdheim–Chester disease (ECD) have a high frequency of BRAF<sup>V600E</sup> mutations and respond to RAF inhibitors. However, detection of mutations in tissue biopsies is particularly challenging in histiocytoses due to low tumor content and stromal contamination. We applied a droplet-digital PCR assay for quantitative detection of the BRAF<sup>V600E</sup> mutation in plasma and urine cell-free (cf) DNA and performed a prospective, blinded study in 30 patients with ECD/LCH. There was 100% concordance between tissue and urinary cfDNA genotype in treatment-naïve samples. cfDNA analysis facilitated identification of previously undescribed KRAS<sup>G12S</sup>-mutant ECD and dynamically tracked disease burden in patients treated with a variety of therapies. These results indicate that cfDNA BRAF<sup>V600E</sup> mutational analysis in plasma and urine provides a convenient and reliable method of detecting mutational status and can serve as a noninvasive biomarker to monitor response to therapy in LCH and ECD.

SIGNIFICANCE: Patients with BRAF<sup>V600E</sup>-mutant histiocytic disorders have remarkable responses to RAF inhibition, but mutation detection in tissue in these disorders is challenging. Here, we identify that analysis of plasma and urinary cfDNA provides a reliable method to detect the BRAF<sup>V600E</sup> mutation and monitor response to therapy in these disorders. Cancer Discov; 5(1); 1–8. © 2014 AACR.

INTRODUCTION

Langerhans cell histiocytosis (LCH) and Erdheim–Chester disease (ECD) are heterogeneous systemic histiocytic disorders characterized by accumulation and infiltration of histiocytes in multiple tissues of the body, leading to organ compromise (1). Although the underlying etiology of these conditions has long been enigmatic, recent investigations have determined that both LCH and ECD are clonal disorders of myeloid-derived precursor cells (2, 3) with a high frequency of somatic BRAF<sup>V600E</sup> mutations (40%–60% of patients; refs. 4–7). Moreover, treatment of patients with BRAF-mutant...
LCH and ECD with the BRAF inhibitor vemurafenib has demonstrated dramatic efficacy, revolutionizing the care of these orphan diseases (8, 9).

The above data underline the importance of accurately identifying BRAF mutational status in patients with systemic LCH and ECD (10, 11). Unfortunately, the scant histiocytic content and marked stromal contamination that are a hallmark of these disorders make mutation detection in tissue biopsies challenging (3, 10). Moreover, the propensity of histiocytic lesions to involve difficult-to-biopsy locations such as the brain, orbits, and right atrium frequently necessitates the use of bone biopsies, further limiting the availability of suitable tumor material for BRAF genotyping (10, 11). Finally, the infiltrative and multifocal nature of these diseases, as well as the absence of a reliable tumor marker, has made evaluation of treatment response challenging.

Given these factors, the use of circulating tumor cell-free DNA (cfDNA) to both identify the BRAFV600E mutation and monitor response to therapy represents a potentially transformative development for these orphan diseases. A recent pilot study of 6 patients with ECD demonstrated that BRAFV600E mutations could be detected in cfDNA (12). However, the concordance of cfDNA BRAF mutational genotype with tissue mutational status is not established in ECD and has never been evaluated in LCH. Moreover, the ability of quantitative cfDNA analysis to detect dynamic changes in BRAFV600E mutation burden during treatment of disease has not been studied. Finally, the use of urine as a source of cfDNA for mutational detection has previously been limited to malignancies of the genitourinary tract and offers significant advantages in sample stability and ease of serial collection.

To evaluate the validity and clinical utility of plasma and urine cfDNA BRAF testing in patients with LCH and ECD, we performed the first-of-a-kind blinded, prospective multi-center study in these disorders.

**RESULTS**

**Cross-Sectional Analysis**

Data from 30 patients (25 ECD and 5 LCH) were analyzed. Patient and sample characteristics are shown in Table 1. Of these 30 patients, initial tissue BRAFV600E genotyping identified 15 patients as mutant, 6 patients as wild-type, and 9 as indeterminate. Bone represented the most common anatomic site of attempted tissue acquisition, accounting for 36.7% of biopsies in this cohort (Table 1).

Urinary cfDNA analysis for detection of the BRAFV600E mutation was performed on all patients, and concordance between cfDNA and tissue DNA mutational results was analyzed. There was 100% concordance between tissue and urinary cfDNA genotype in samples from treatment-naive patients. Urinary BRAFV600E cfDNA values obtained from any time point in therapy identified 16 patients as mutant and 14 as wild-type (kappa = 0.88; 95% CI, 0.66–1.0; Supplementary Table S1). This resulted in a sensitivity of urinary cfDNA BRAFV600E detection of 92.9%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 85.7% (all compared to BRAFV600E detection from tissue biopsy). Overall, urinary cfDNA analysis identified 2 patients as being BRAFV600E mutant who were not known to have the BRAF mutation previously. Subsequent tissue biopsy was performed in these patients and identified the BRAFV600E mutation, allowing both patients to enroll in an ongoing phase II study of vemurafenib for patients with BRAFV600E-mutant ECD and LCH (NCT01524978). Thus, tissue-based genotyping resulted in 21 of 30 (70%) patients with definite BRAFV600E status compared to 30 of 30 (100%) using urinary cfDNA (Fig. 1A).

Urinary cfDNA analysis failed to detect the BRAFV600E mutation in 1 of 15 (6.7%) patients positive by tissue biopsy. Of note, the urine and plasma utilized for cfDNA analysis in this case were sampled while the patient was in active treatment with a BRAF inhibitor with ongoing reduction in
disease burden, whereas the tissue genotyping was performed before treatment. When considering only urinary or plasma samples obtained from treatment-naïve patients, there was a 100% concordance between tissue and urinary cfDNA genotype from urine and plasma (Supplementary Fig. S1A and B).

Plasma cfDNA and urinary cfDNA were obtained at the same time point in 19 of 30 (63.3%) patients. Results from plasma cfDNA for identifying the \textit{BRAF} \textit{V600E} mutation were comparable to urinary cfDNA results (Supplementary Table S2 and Supplementary Fig. S1). Plasma cfDNA analysis identified 9 patients as mutant and 10 as wild-type. \textit{BRAF} \textit{V600E} geno-type as determined by a urinary and plasma cfDNA assay was concordant for all samples from the 19 patients with both tests (\( n = 26 \) tests), except one (which was obtained from a patient during RAF inhibitor therapy; 96% concordance). The quantitative \textit{BRAF} \textit{V600E} mutant:\textit{BRAF} wild-type ratio was significantly higher in the cfDNA from plasma as well as urine in those patients whose tissue was \textit{BRAF} \textit{V600E} versus wild-type (\( P = 0.0005 \) and 0.002, respectively; Fig. 1B and C).

**Longitudinal Assessment of \textit{BRAF} \textit{V600E} cfDNA Burden**

Comparing cfDNA \textit{BRAF} \textit{V600E}:\textit{BRAF} wild-type ratios of pretreatment versus BRAF inhibitor–treated \textit{BRAF} \textit{V600E}-mutant patients, a significant decrease in the \textit{BRAF} \textit{V600E}:\textit{BRAF} wild-type ratio was seen with therapy (\( P < 0.0001 \); Fig. 2A). Serial samples on 13 \textit{BRAF} \textit{V600E}-mutant patients were available, 10 of whom were treated with a BRAF inhibitor. In all patients treated with a BRAF inhibitor, serial urinary cfDNA analysis revealed progressive decrements in the \textit{BRAF} \textit{V600E} allele burden (Fig. 2B). Weekly serial urinary cfDNA analysis throughout the course of BRAF inhibitor therapy revealed that the decline in mutant cfDNA burden in response to BRAF inhibitors was consistent with radiographic disease improvement (Fig. 3A and B).

Serial cfDNA \textit{BRAF} \textit{V600E} burden was also assessed in two patients treated with anakinra, an IL1 receptor antagonist commonly used as an off-label treatment for ECD (13).
Interestingly, treatment with anakinra also reduced the \( \text{BRAF}^{\text{V600E}} \)-mutant allele burden (Fig. 3C). Anakinra was subsequently discontinued in one patient, and within 7 days the urinary cfDNA \( \text{BRAF}^{\text{V600E}} \) allele burden increased. Vemurafenib was then initiated in this patient, and once again \( \text{BRAF}^{\text{V600E}} \) allele burden as assessed in cfDNA decreased within 2 weeks of BRAF inhibitor therapy.

In at least one patient for whom successful RAF inhibitor therapy was discontinued due to toxicity, urinary cfDNA \( \text{BRAF}^{\text{V600E}} \) burden increased after vemurafenib discontinuation, which mirrored radiographic evidence of disease recurrence (Fig. 3D).

Identification of a KRAS Mutation in a BRAF\(^{V600E}\) Wild-Type Patient

Of the patients enrolled in this study, 56.7% (17 of 30) were identified as having a \( \text{BRAF}^{\text{V600E}} \) mutation based on either tissue genotyping and/or cfDNA analysis. In addition to prevalent \( \text{BRAF}^{\text{V600E}} \) mutations in these diseases, recurrent \( \text{RAS} \) mutations have also been recently identified in ECD (14), and therefore a noninvasive method of diagnosing somatic mutations in patients with \( \text{BRAF} \) wild-type ECD is of potential value. One \( \text{BRAF} \) wild-type patient here was found to have a \( \text{KRAS}^{\text{G12S}} \) mutation in tissue material taken from a cardiac ECD lesion (Supplementary Fig. S2A–D). This mutation was also found to be present by cfDNA analysis in both plasma and urine (Supplementary Fig. S2E and Supplementary Table S3). Although NRAS mutations have been reported in ECD (15), \( \text{KRAS} \) mutations have never previously been reported in these disorders.

### DISCUSSION

This study demonstrates the utility of circulating cfDNA for reliably detecting actionable alterations and monitoring response to therapy in patients with histiocytic disorders. We identified a high correlation of tissue mutational genotype with urine and plasma cfDNA mutational status, establishing the utility of cfDNA assessment of \( \text{BRAF}^{\text{V600E}} \) mutations in patients with LCH and ECD. Moreover, quantitative \( \text{BRAF}^{\text{V600E}} \) cfDNA allele burden changed dynamically with therapy and mirrored radiographic evaluation of disease. These findings have potentially important implications for the initial diagnostic workup and serial monitoring of these rare disorders.

We found that 30% of patients (9 of 30) had an indeterminate \( \text{BRAF} \) mutation result from tumor tissue despite concerted genotyping efforts. This high proportion of patients with unknown tissue biopsy genotype underscores the substantial difficulty in identifying tumor genotype information in patients with histiocytic disorders. The high proportion of \( \text{BRAF} \) genotyping test failures here likely relates to the frequent use of bone as a site of biopsy in these disorders. Eight of the 9 (88.9%) patients with an initial unknown \( \text{BRAF} \) tissue
Figure 3. Dynamic monitoring of serial urinary cfDNA BRAF**V600E** mutant allele burden in patients with systemic histiocytosis. A, gadolinium-enhanced T1 MRI images of ECD involvement of brain (green arrows), and 2[**18**F]fluoro-2-deoxy-D-glucose (FDG)-PET images of disease in the right atrium (asterisk) and testes (asterisk), pre-dabrafenib, and after 2 months of dabrafenib. B, urinary BRAF**V600E** cfDNA results throughout this same patient’s therapy. C, urinary BRAF**V600E** cfDNA results of a patient with ECD treated with anakinra followed by a period of treatment cessation and then initiation of vemurafenib. D, maximal intensity projection images of FDG–PET scan images (top) demonstrating tibial infiltration by ECD pre-vemurafenib (left scan), following 10 weeks of vemurafenib (middle scan), and then 16 weeks after vemurafenib discontinuation (right scan) in a patient with ECD with accompanying urinary cfDNA results for each time point (bottom).
Assessment of Cell-Free BRAF\textsuperscript{V600E} DNA in Histiocytes

**Methods**

**Patients**

Between January 2013 and June 2014, 30 consecutive patients with LCH and ECD seen at the Memorial Sloan Kettering Cancer Center (MSKCC) and The MD Anderson Cancer Center (MDACC) were enrolled in the study.

Tissue biopsies were performed as part of routine clinical care, with the site of biopsy based on radiographic and/or clinical assessment of disease involvement. Ten milliliters of blood and between 60 and 120 mL of urine were collected at each time point. Plasma was separated from blood samples using standard techniques. All samples were deidentified, and operators performing plasma and urine cfDNA analyses were blinded to the tissue genotype and clinical characteristics of all patients.

Institutional review boards at both MSKCC and MDACC approved the study protocol.

Of note, 6 plasma and 6 urinary cfDNA values that were previously reported in a pilot proof-of-concept study (12) were not included in the current study or data analysis.

**Tissue Mutational Genotyping**

Initial BRAF tissue mutation testing was performed by a variety of methods as part of routine care in Clinical Laboratory Improvements Amendments (CLIA)-certified molecular diagnostic laboratories at MSKCC, MDACC, or the institution from which the patient was initially referred. Tissue with a BRAF\textsuperscript{V600E} mutation identified as part of these analyses was considered positive. For tissue to be considered negative for the BRAF\textsuperscript{V600E} mutation for the purposes of this analysis, it was required to undergo further testing by a high-sensitivity assay, either Sanger sequencing with locked nuclear acid (LNA) clamping or next-generation sequencing. Sequencing with LNA was performed according to previously published procedures (20) and had a limit of detection of 0.5% mutant alleles. Massively parallel sequencing was performed by Foundation Medicine, Inc., using previously published methodologies (21), with a minimum coverage of <500. In patients for whom initial diagnostic tissue was insufficient for genotyping, additional biopsies were attempted as deemed appropriate by the treating physician. Patients were considered tumor BRAF indeterminate if they met one of the following criteria: (i) inadequate tumor material for genotyping despite multiple biopsy attempts, (ii) declined repeated biopsy for the purpose of genotyping, and (iii) tissue genotyping was ordered but no result was obtained due to failure of the tumor material to meet technical requirements. Next-generation sequencing of genomic DNA from one BRAF wild-type tumor tissue biopsy was performed on a panel of 30 genes (ASXL1, CBL, CEBPA, DNMT3A, ETV6, EZH2, FLT3, HRAS, IDH1/2, JAK1/2/3, KIT, KRAS, MPL, NPM1, NRAS, PHF6, PTPN11, RUNX1, SF3B1, SH2B3, SUZ12, TET1–3, TP53, TYK2, and WTI) by MiSeq at a depth of >500x.

**Plasma and Urine cfDNA Extraction and Analyses**

Plasma cfDNA was isolated using the QiAamp Circulating Nucleic Acid Kit (QiaGen) according to the manufacturer’s instructions. Urine cfDNA was isolated as previously described (12).

Urine and plasma cfDNA were quantified by a droplet-digital PCR (ddPCR; QX-100, BioRad) assay to a 44-bp amplicon of RNase P, a single-copy gene, as previously described (12). Quantified DNA up to 60 ng was used for mutation detection of BRAF\textsuperscript{V600E} by ddPCR and KRAS mutations at codons 12 and 13 of exon 2 by massively parallel sequencing.

The combined use of tissue and cfDNA genotyping analyses also allowed us to identify a KRAS mutation in a BRAF wild-type ECD patient (a mutation not previously described in ECD). Future interrogation of RAS mutations in tumor biopsies and cfDNA from patients with BRAF wild-type histiocytic disorders may provide an additional somatic mutational biomarker and therapy options in this patient population.

Overall, these data suggest that monitoring of BRAF\textsuperscript{V600E} mutations in the cfDNA of patients with histiocytic disorders provides a reliable and convenient noninvasive method to detect BRAF\textsuperscript{V600E} mutations and assess treatment response in these unique disorders.
For **BRAF**<sup>V600E</sup> mutation detection, a two-step PCR assay targeting a very short (31 bp) amplicon was employed to enhance detection of rare mutant alleles in cfDNA. The first-step amplification was done with two primers flanking the **BRAF**<sup>V600E</sup> locus, where both primers contain noncomplementary 5’ tags that hybridize to second-round primers. A complementary blocking oligonucleotide suppressed wild-type **BRAF** amplification, achieving enrichment of the mutant **BRAF**<sup>V600E</sup> sequence in this step. The second step entailed a duplex ddPCR reaction using FAM (**BRAF**<sup>V600E</sup>) and VIC (wild-type **BRAF**) TaqMan probes to enable differentiation of mutant versus wild-type quantification, respectively. The RainDrop ddPCR platform (RainDance) was used for PCR droplet separation, fluorescent reading, and counting droplets containing mutant sequence, wild-type sequence, or unreacted probe.

For each patient sample, the assay identified **BRAF**<sup>V600E</sup> mutation fragments detected as a percentage of detected wild-type **BRAF**. As previously published, thresholds for the **BRAF** assay were initially developed by evaluating a training set of urinary cfDNA from patients with **BRAF**<sup>V600E</sup> metastatic cancer (positives) and healthy volunteers (negatives) using a classification tree that maximized the true-positive and true-negative rates (12, 22). Using this training set, a double threshold approach with an indeterminate range between not detected and detected was estimated, yielding two threshold values (<0.05 for not detected; 0.05–0.107 indeterminate; >0.107 detected; ref 12). For this current study, however, the assay was simplified to a dichotomous classifier by combining both indeterminate and negative ranges as “not detected,” yielding a single cutoff of ≤0.107 for not detected and >0.107 detected. A single cutoff point was preselected to evaluate the performance of this assay within this cohort for false-positive and false-negative rates for the detection of **BRAF**<sup>V600E</sup> (this was chosen because definitions of sensitivity and specificity are not compatible with a classifier containing an indeterminate range).

For plasma detection, wild-type **BRAF** patients with metastatic cancer (13 plasma samples) were used to determine a threshold for detection of **BRAF**<sup>V600E</sup> mutations. The **BRAF**<sup>V600E</sup> values for this wild-type **BRAF** population were normally distributed, and therefore a cutoff point equivalent to 3 SDs (0.021%) above the mean of wild-type **BRAF** controls (0.031%) or >0.094% mutant to wild-type was considered positive for **BRAF**<sup>V600E</sup> (12).

For **KRAS** mutation detection (G12A/C/D/R/S/V, G13D), a two-step PCR assay similar to that described for **BRAF**<sup>V600E</sup> was employed with an initial 31-bp targeted region, except that during the second round, flanking primers were used to add patient-specific barcodes and adaptor sequences necessary for massively parallel DNA sequencing per manufacturer’s instructions (MiSeq; Illumina). Sequence reads were filtered for quality (Q score > 20) and verified as matching the target sequence (no more than 3 mismatches permitted outside the mutation region). For each sample, **KRAS**-mutant sequences were tallied and the percentage of mutant was computed. For the **KRAS** assay, the distribution of background signal in the wild-type population was observed not to conform to a normal distribution. To be consistent with the plasma **BRAF** assay approach for computing the threshold (mean ± 3 SD), the median and median absolute deviation of a **KRAS** wild-type population was used to produce a “robust” z-score, and a cutoff of greater than 3 z-scores above the median mutant signal count of the population (or >0.02%) was determined to be a positive result (23). This approach is approximately equal to the mean ± 3 SD threshold when the data are normally distributed (data not shown).

**Statistical Analysis**

Statistical analyses were performed with GraphPad Prism V5.0 for Macintosh (GraphPad Prism Software). The Mann–Whitney U test was used to compare **BRAF**<sup>V600E</sup> mutant:**BRAF** wild-type ratios determined by cfDNA analysis in patients identified as **BRAF** wild-type based on tissue biopsy versus those identified as **BRAF**<sup>V600E</sup> mutant based on tissue biopsy. In addition, the Mann–Whitney U test was also used to compare **BRAF**<sup>V600E</sup> mutant:**BRAF** wild-type ratios obtained from urinary cfDNA pretreatment with vemurafenib versus urinary cfDNA **BRAF**<sup>V600E</sup> mutant:**BRAF** wild-type ratios obtained following initiation of therapy with vemurafenib. Concordance of tissue, plasma, and urinary assessment of **BRAF**<sup>V600E</sup> mutational detection was performed by calculating the kappa coefficient. A two-tailed P value of <0.05 was considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

C.R.T. Vibat is Senior Director, Translational Science and Clinical Affairs, at Trovagene and has ownership interest (including patients) in the same. M.E. Lacouture is a consultant/advisory board member for Roche, GlaxoSmithKline, Genentech, and AstraZeneca. F. Janku reports receiving commercial research grants from Trovagene, Biocartis, and Transgenic.

One of the Editors-in-Chief of Cancer Discovery is an author of this article. In keeping with the AACR’s editorial policy, the paper was peer reviewed and an AACR journal editor not affiliated with Cancer Discovery rendered the decision concerning acceptability.

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