Prospective Blinded Study of \( \text{BRAF}^{V600E} \) Mutation Detection in Cell-Free DNA of Patients with Systemic Histiocytic Disorders

David M. Hyman\(^1\), Eli L. Diamond\(^2\), Cecile Rose T. Vibat\(^3\), Latifa Hassaine\(^4\), Jason C. Poole\(^5\), Minal Patel\(^6\), Veronica R. Holley\(^7\), Goran Cabrilo\(^8\), Timothy T. Lu\(^9\), Maria E. Arcila\(^6\), Young Rock Chung\(^7\), Raajit Rampal\(^4\), Mario E. Lacouture\(^8\), Neal Rosen\(^9\), Funda Meric-Bernstam\(^1\), José Baselga\(^1\), Razelle Kurzrock\(^10\), Mark G. Erlander\(^3\), Filip Janku\(^5\), and Omar Abdel-Wahab\(^4\,\(^7\)

1 Developmental Therapeutics Unit, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. 2 Department of Neurology, Memorial Sloan Kettering Cancer Center, New York, New York. 3 Trovagene, Inc., San Diego, California. 4 Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. 5 Department of Investigational Cancer Therapeutics (Phase I Program), The MD Anderson Cancer Center, Houston, Texas. 6 Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York. 7 Human Oncology and Pathogenesis Program, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. 8 Dermatology Service, Memorial Sloan Kettering Cancer Center, New York, New York. 9 Program in Molecular Pharmacology, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. 10 Moores Cancer Center, University of California, San Diego, La Jolla, California.

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D.M. Hyman, E.L. Diamond, F. Janku, and O. Abdel-Wahab contributed equally to this article.

Corresponding Author: Omar Abdel-Wahab, Memorial Sloan Kettering Cancer Center, 519 Zuckerman Research Building, 415 East 68th Street, New York, NY 10065. Phone: 347-821-1769; Fax: 646-422-0856; E-mail: abdelwao@mskcc.org
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ABSTRACT
Patients with Langerhans cell histiocytosis (LCH) and Erdheim–Chester disease (ECD) have a high frequency of \( \text{BRAF}^{V600E} \) 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 \( \text{BRAF}^{V600E} \) 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 \( \text{KRAS}^{G12S} \)-mutant ECD and dynamically tracked disease burden in patients treated with a variety of therapies. These results indicate that cfDNA \( \text{BRAF}^{V600E} \) 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 \( \text{BRAF}^{V600E} \)-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 \( \text{BRAF}^{V600E} \) mutation and monitor response to therapy in these disorders. Cancer Discov; 5(1); 64–71. ©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 \( \text{BRAF}^{V600E} \) mutations (40%–60% of patients; refs. 4–7). Moreover, treatment of patients with \( \text{BRAF} \)-mutant
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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 histiocyte 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} 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 BRAF\textsuperscript{V600E} detection from tissue biopsy). Overall, urinary cfDNA analysis identified 2 patients as being BRAF\textsuperscript{V600E} mutant who were not known to have the BRAF mutation previously. Subsequent tissue biopsy was performed in these patients and identified the BRAF\textsuperscript{V600E} mutation, allowing both patients to enroll in an ongoing phase II study of vemurafenib for patients with BRAF\textsuperscript{V600E}-mutant ECD and LCH (NCT01524978). Thus, tissue-based genotyping resulted in 21 of 30 (70%) patients with definitive BRAF status compared to 30 of 30 (100%) using urinary cfDNA (Fig. 1A).

Urinary cfDNA analysis failed to detect the BRAF\textsuperscript{V600E} 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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Median age, (range), y</td>
<td>56 (9–75)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (53.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (46.7%)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>ECD</td>
<td>25 (83.3%)</td>
</tr>
<tr>
<td>LCH</td>
<td>5 (16.7%)</td>
</tr>
<tr>
<td>Sites of tissue biopsy, % of cohort (number of patients)(^a)</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>11 (36.7%)</td>
</tr>
<tr>
<td>Abdominal soft tissue (e.g., retroperitoneum)</td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>Skin</td>
<td>6 (20.0%)</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>5 (16.7%)</td>
</tr>
<tr>
<td>Cardiac tissue</td>
<td>2 (6.7%)</td>
</tr>
<tr>
<td>Median number of organ sites involved (range)(^b)</td>
<td></td>
</tr>
<tr>
<td>ECD</td>
<td>3 (0–11)</td>
</tr>
<tr>
<td>LCH</td>
<td>2 (1–4)</td>
</tr>
<tr>
<td>Median number of urine collections (per patient, range)</td>
<td>2 (1–8)</td>
</tr>
<tr>
<td>Median number of plasma collections per patient (range)</td>
<td>1 (0–7)</td>
</tr>
<tr>
<td>Number of paired urine and plasma collections</td>
<td>27 (90%)</td>
</tr>
<tr>
<td>Number of patients with initial sample acquired while off therapy</td>
<td>26 (86.7%)</td>
</tr>
</tbody>
</table>

\(^a\)Several individual patients underwent more than one biopsy.

\(^b\)All patients with LCH had multisystem disease with risk-organ involvement (11).

\(^c\)Refers to the number of therapies before the first cfDNA analysis.
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 genotyping 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 \( \text{BRAF}^{V600E} \) 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. \( \text{BRAF} \) genotype as determined by a urinary and plasma cfDNA assay was concordant for all samples from the 19 patients with both tests (26 tests), except one (which was obtained from a patient during RAF inhibitor therapy; 96% concordance). The quantitative \( \text{BRAF}^{V600E} \) mutant: \( \text{BRAF} \) wild-type ratio was significantly higher in the cfDNA from plasma as well as urine in those patients whose tissue was \( \text{BRAF}^{V600E} \) versus wild-type (\( P = 0.0005 \) and 0.002, respectively; Fig. 1B and C).

### Figure 1. \( \text{BRAF}^{V600E} \) mutant allele burden in cfDNA of urine and plasma based on \( \text{BRAF}^{V600E} \) tissue genotype result

A pie chart representation of \( \text{BRAF}^{V600E} \) mutational genotype as determined by initial tissue biopsy (left) or urinary cfDNA analysis (right). Results were recorded as \( \text{BRAF}^{V600E} \) mutant (yellow), \( \text{BRAF}^{V600E} \) wild-type (white), or indeterminate (gray).

B, ratio of \( \text{BRAF}^{V600E}:\text{BRAF} \) wild-type in urinary cfDNA of patients based on \( \text{BRAF} \) mutational status as determined from tissue biopsy (\( \text{BRAF}^{V600E} \) mutant, \( \text{BRAF} \) wild-type, or \( \text{BRAF} \) indeterminate). Lines and error bars for \( \text{BRAF}^{V600E} \)-mutant and \( \text{BRAF} \) wild-type patients represent mean ± SEM. C, ratio of \( \text{BRAF}^{V600E}:\text{BRAF} \) wild-type in plasma cfDNA of patients based on \( \text{BRAF} \) mutational status as determined from tissue biopsy. Each point represents a single test result from the initial assessment of the \( \text{BRAF}^{V600E} \)-BRAF wild-type allelic ratio in cfDNA. Dotted points, samples collected during therapy. Red dashed line, the cutoff point defining a positive versus a negative cfDNA result.

### Longitudinal Assessment of \( \text{BRAF}^{V600E} \) cfDNA Burden

Comparing cfDNA \( \text{BRAF}^{V600E}:\text{BRAF} \) wild-type ratios of pre-treatment versus BRAF inhibitor-treated \( \text{BRAF}^{V600E} \)-mutant patients, a significant decrease in the \( \text{BRAF}^{V600E}:\text{BRAF} \) wild-type ratio was seen with therapy (\( P < 0.0001 \); Fig. 2A). Serial samples on 13 \( \text{BRAF}^{V600E} \)-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 \( \text{BRAF}^{V600E} \)-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 \( \text{BRAF}^{V600E} \) burden was also assessed in two patients treated with anakinra, an IL1 receptor antagonist commonly used as an off-label treatment for ECD (13).
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Interestingly, treatment with anakinra also reduced the $BRAF^{V600E}$-mutant allele burden (Fig. 3C). Anakinra was subsequently discontinued in one patient, and within 7 days the urinary cfDNA $BRAF^{V600E}$ allele burden increased. Vemurafenib was then initiated in this patient, and once again $BRAF^{V600E}$ 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 $BRAF^{V600E}$ 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 $BRAF^{V600E}$ mutation based on either tissue genotyping and/or cfDNA analysis. In addition to prevalent $BRAF^{V600E}$ mutations in these diseases, recurrent RAS mutations have also been recently identified in ECD (14), and therefore a noninvasive method of diagnosing somatic mutations in patients with $BRAF$ wild-type ECD is of potential value. One $BRAF$ wild-type patient here was found to have a $KRAS^{G12S}$ 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), 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 $BRAF^{V600E}$ mutations in patients with LCH and ECD. Moreover, quantitative $BRAF^{V600E}$ 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 $BRAF$ 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 $BRAF$ 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 $BRAF$ tissue
**Figure 3.** Dynamic monitoring of serial urinary cfDNA BRAF<sup>V600E</sup>-mutant allele burden in patients with systemic histiocytosis. **A,** gadolinium-enhanced T1 MRI images of ECD involvement of brain (green arrows), and 18-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<sup>V600E</sup> cfDNA results throughout this same patient’s therapy. **C,** urinary BRAF<sup>V600E</sup> 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).
genotyping status had biopsies from bone. The molecular assessment of bony lesions is challenging, as morphologic assessment requires decalcification procedures that often render the tissue unsuitable for molecular testing. Furthermore, aspirates of these lesions often yield suboptimal material for testing, with findings of nonspecific inflammation and/or fibrosis and low histiocyte content. Of the 9 patients with indeterminate BRAF genotype from tissue biopsy, cfDNA testing identified BRAF mutations in 2 patients. These results have immediate therapeutic implications.

In addition to the use of cfDNA for establishing the initial presence or absence of BRAFV600E mutations, serial measurements of BRAFV600E-mutant allele burden on a variety of therapies revealed the utility of cfDNA analysis for dynamically monitoring response to both immunomodulatory and BRAF inhibitor therapy in these disorders. Assessment of treatment response has been an obstacle in the treatment of adult patients with histiocytic disorders, as radiographic assessments of response do not accurately characterize the wide spectrum of anatomic sites and lesion types characteristic of these disorders. Moreover, no formal criteria for the assessment of treatment response exist for adult patients with LCH and ECD (10). Thus, these data support incorporation of urinary and/or plasma cfDNA allele burden as a potential surrogate marker for clinical benefit in future clinical trials and standard of care for patients with histiocytic disorder.

It is important to note that the rate of decline in the BRAF-mutant allele burden in urinary and plasma cfDNA was variable between patients, underlining the need for multiple serial assessments of allele burden following initiation of therapy. Also, given that quantitative cfDNA BRAFV600E mutation detection mirrored response to multiple therapeutic modalities, it is likely that cfDNA detection of BRAF mutations will serve as a good marker of disease burden not only in response to RAF-targeted therapy but also across a range of therapeutic agents commonly utilized in these disorders.

The use of urine as the source of cfDNA as reported here particularly facilitated routine serial monitoring of BRAFV600E allele burden. Although somatic mutation detection has previously been performed in the cfDNA of patients with cancer, nearly all prior studies utilizing urinary cfDNA in cancer were restricted to patients with genitourinary malignancies (16–18). However, urinary cfDNA detection of BRAFV600E mutations mirrored closely the results from plasma cfDNA analysis here. Moreover, as shown in Fig. 3, urinary samples for cfDNA could be obtained on a weekly basis, allowing for disease monitoring on an outpatient basis without the need for phlebotomy or other medical procedures. Previous studies indicate that DNA in urine can be stabilized for at least 9 days (18), whereas plasma requires processing within 6 hours for accurate assessment of cfDNA (19).

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 BRAFV600E mutations in the cfDNA of patients with histiocytic disorders provides a reliable and convenient noninvasive method to detect BRAFV600E mutations and assess treatment response in these unique disorders.

### 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 BRAFV600E mutation identified as part of these analyses was considered positive. For tissue to be considered negative for the BRAFV600E 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 ×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) tumor 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, PTEK, RUNX1, SF3B1, SH2B3, SUZ12, TET1–3, TP53, TTK, and WT1) by MiSeq at a depth of ×500×.

#### Plasma and Urine cfDNA Extraction and Analyses

Plasma cfDNA was isolated using the QIAMP 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 BRAFV600E by ddPCR and KRAS mutations at codons 12 and 13 of exon 2 by massively parallel sequencing.
For BRAFV600E 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 was amplified with two primers flanking the BRAFV600E 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 BRAFV600E sequence in this step. The second step entailed a duplex ddPCR reaction using FAM (BRAFV600E) and VIC (wild-type BRAF) TaqMan probes to enable differentiation of mutant versus wild-type quantification, respectively. The RainDrop ddPCR platform (Rain-Dance) 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 BRAFV600E 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 BRAFV600E-mutated 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 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 for 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 BRAFV600E (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 BRAFV600E mutations. The BRAFV600E 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 BRAFV600E (12).

For KRAS mutation detection (G12A/C/D/R/S/V, G13D), a two-step PCR assay similar to that described for BRAFV600E 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 4 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 BRAFV600E mutant:BRAF wild-type ratios determined by cfDNA analysis in patients identified as BRAF wild-type based on tissue biopsy versus those identified as BRAFV600E mutant based on tissue biopsy. In addition, the Mann–Whitney U test was also used to compare BRAFV600E mutant:BRAF wild-type ratios obtained from urinary cfDNA pretreatment with vemurafenib versus urinary cfDNA BRAFV600E mutant:BRAF wild-type ratios obtained following initiation of therapy with vemurafenib. Concordance of tissue, plasma, and urinary assessment of BRAFV600E 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. Vihat is Senior Director, Translational Science and Clinical Affairs, at Trovagen and has ownership interest (including patents) 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 Trovagen, Biocartis, and Transgenomic.

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.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Hyman, E.L. Diamond, L. Hassaine, M. Patel, M.E. Arcila, Y.R. Chung, M.E. Lacouture, F. Janku, O. Abdel-Wahab


Writing, review, and/or revision of the manuscript: D.M. Hyman, E.L. Diamond, C.R.T. Vihat, J.C. Poole, M.E. Arcila, M.E. Lacouture, N. Rosen, F. Meric-Bernstam, J. Baselga, R. Kurzrock, M.G. Erlander, F. Janku, O. Abdel-Wahab

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.M. Hyman, C.R.T. Vihat, M. Patel, V.R. Holley, T.T. Lu, M.E. Lacouture, F. Janku, O. Abdel-Wahab

Study supervision: D.M. Hyman, M.G. Erlander, F. Janku, O. Abdel-Wahab

Other (handled collection and processed blood and urine samples): G. Cabrillo

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