ABSTRACT

Kinase inhibitors are accepted treatment for metastatic melanomas that harbor specific driver mutations in BRAF or KIT, but only 40% to 50% of cases are positive. To uncover other potential targetable mutations, we conducted whole-genome sequencing of a highly aggressive BRAF (V600) and KIT (W557, V559, L576, K642, and D816) wild-type melanoma. Surprisingly, we found a somatic BRAF L597R mutation in exon 15. Analysis of BRAF exon 15 in 49 tumors negative for BRAF V600 mutations as well as driver mutations in KIT, NRAS, GNAQ, and GNA11, showed that two (4%) harbored L597 mutations and another two involved BRAF D594 and K601 mutations. In vitro signaling induced by L597R/S/Q mutants was suppressed by mitogen-activated protein (MAP)/extracellular signal–regulated kinase (ERK) kinase (MEK) inhibition. A patient with BRAF L597S mutant metastatic melanoma responded significantly to treatment with the MEK inhibitor, TAK-733. Collectively, these data show clinical significance to BRAF L597 mutations in melanoma.

SIGNIFICANCE: This study shows that cells harboring BRAF L597 mutants are sensitive to MEK inhibitor treatment, providing a rationale for routine screening and therapy of BRAF L597-mutant melanoma.

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INTRODUCTION

Melanoma is a malignant tumor of melanocytes that caused nearly 9,000 deaths in the United States in 2011 (1). Recently, the kinase inhibitors vemurafenib and, to a lesser extent, imatinib have become standard treatments for patients whose metastatic malignant melanomas harbor specific driver mutations in BRAF (V600E; ref. 2) or KIT (exons 9, 11, 13, 17, and 18) (3). Among the many mutations identified, we focused on the biological and clinical significance of a confirmed somatic mutation at the BRAFV600E codon. Our findings have direct therapeutic implications for patients with metastatic melanoma.

RESULTS

Identification of a BRAFL597R Mutation by Whole-Genome Sequencing

A 75-year-old white man presented with an ulcerated right ear melanoma that was widely excised with uninvolved sentinel lymph nodes. Four months later, he developed local recurrence and underwent additional surgery as well as postoperative radiation. This specimen was negative for the BRAFV600E (c.1799T>A) mutation using an allele-specific PCR assay, and negative for KIT exons 9, 11, 13, 17, and 18 by PCR-based methods. Twelve months later, the patient developed widespread metastasis and required a palliative thyroidectomy. He died 13 days later with both cardiac and brain involvement.

To identify potential driver mutations in his tumor using an unbiased genome-wide approach, we conducted WGS of DNA from his metastatic lesion, along with DNA from matched blood (see Supplementary Methods for case description and variation calling). A subset of the single-nucleotide polymorphisms (SNP), insertions, and deletions were validated by whole-genome sequencing (WGS) of a tumor/normal pair from a patient with a highly aggressive BRAF metastatic melanoma. Among the many mutations identified, we focused on the biological and clinical significance of a confirmed somatic mutation at the BRAFV600E codon. Our findings have direct therapeutic implications for patients with metastatic melanoma.

Frequency of BRAFL597 and Other Exon 15 Mutations in “BRAF Wild-type” Melanoma

Both BRAF L597 and V600 are encoded by exon 15. To determine how many exon 15 mutations might be overlooked by assessing only the V600 position, we analyzed the mutational status of the entire coding exon in 49 additional tumor samples negative for mutations at V600 as well as for recurrent mutations in NRAS (G12/13, Q61), KIT (W557, V559, L576, K642, and D816), GNAQ (Q209), and GNA11 (Q209; ref. 4). Two (4%) additional tumors had BRAFmutant mutations (c.1790T>A, p.L597Q; Table 1). A third tumor harbored a BRAFV600E mutation (c.1790T>A), whereas a fourth had a D594N mutation (c.1780G>A; Table 1). The BRAF c.1789_1790CT>TGC mutation was confirmed to be in cis by cloning and sequencing of the PCR product (data not shown). Thus, 8% of “pan-negative” cases harbored additional non-V600 BRAF exon 15 mutations.

Table 1. Mutations detected in BRAF exon 15 in 49 SNaPshot screen “pan-negative” melanomas

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<tr>
<th>Sample</th>
<th>Nucleotide</th>
<th>Amino acid</th>
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<tbody>
<tr>
<td>1</td>
<td>c.1801A&gt;G</td>
<td>p.K601E</td>
</tr>
<tr>
<td>2</td>
<td>c.1790T&gt;A</td>
<td>p.L597Q</td>
</tr>
<tr>
<td>3</td>
<td>c.1789_1790CT&gt;TGC</td>
<td>p.L597Q</td>
</tr>
<tr>
<td>4</td>
<td>c.1780G&gt;A</td>
<td>p.D594N</td>
</tr>
</tbody>
</table>

*Samples considered melanoma SNaPshot screen “pan-negative” were those that did not have mutations in BRAF (V600E), NRAS (G12/13, Q61), KIT (W557, V559, L576, K642, and D816), CTNNB1 (S37/45), GNAQ (Q209), and GNA11 (Q209).
**Figure 1.** Detection and validation of the BRAF L597R mutation. **A,** alignment of next-generation sequence reads displaying the BRAF L597R mutation compared with the reference human genome. The region shown represents base pairs 140,453,089-140,453,201 on chromosome 7. Seventy-two reads overlap the mutant position (chr7:140,453,145), including 47 reverse (in red) and 25 forward (in blue) strands, respectively. **B,** sequencing chromatograms show the presence of a heterozygous BRAF L597R mutation in the tumor but not in the matched blood. Arrows indicate the position of the mutant or wild-type peaks.
Objective Radiographic Response to MEK Inhibitor Therapy in a Patient with a Metastatic Melanoma with a \(\text{BRAF}^{L597}\) Mutation

A 69-year-old patient with a metastatic melanoma who had previously received therapy with dacarbazine chemotherapy was enrolled in a phase I trial testing of the allosteric MEK inhibitor TAK-733 (8). After 2 cycles of therapy, the patient was noted to have a partial radiographic response with a decrease of 31% in the sum of maximum diameters of target metastatic lesions in the liver and spleen and as of this submission remains progression free at more than 24 weeks (Fig. 3 and Supplementary Table S9). Follow-up sequencing analysis of DNA from the patient’s tumor revealed a somatic \(\text{BRAF}^{L597}\) (c.1789_1790CT > TC) mutation (Supplementary Fig. S10). These data validate the notion that \(\text{BRAF}^{L597}\)-mutated melanomas are sensitive to MEK inhibitors in patients.

DISCUSSION

Inhibitors of mutant BRAF and the mitogen-activated protein kinase (MAPK) pathway have been shown to improve the overall survival of patients with melanomas that harbor recurrent genetic alterations involving \(\text{BRAF}^{V600E}\) (c.1799T > A; refs. 2, 9). Here, WGS of a tumor–normal pair revealed that a \(\text{BRAF}\) wild-type tumor harbored an unexpected \(\text{BRAF}^{L597}\) mutation occurring in exon 15. Through analysis of additional tumors negative for recurrent mutations in \(\text{BRAF}^{V600E/K/M/R/D}\) as well as \(\text{NRAS}^{G12/13, Q61}\), \(\text{KIT}^{W557, V559, L576, K642, and D816}\), \(\text{GNAQ}^{Q209}\), and \(\text{GNA11}^{Q209}\); ref. 4), we show that \(\text{BRAF}\) exon 15 mutations not involving the V600 codon are relatively common [4 of 49 samples (8%), including 2 \(\text{L597}\) mutations], consistent with other studies in melanoma (10). We further show in vitro that signaling induced by ectopic expression of \(\text{BRAF}^{L597}\) and \(\text{K601}\) mutants is suppressed by MEK and BRAF inhibition.

Figure 3. Computed tomographic images from a patient with \(\text{BRAF}^{L597}\)-mutant metastatic melanoma responding to therapy with the MEK inhibitor TAK-733. Arrows indicate the tumor in the liver (left) and spleen (right) at baseline (A) and after 4 cycles of treatment (B).
Finally, we report a case of a patient whose BRAF<sup>L597</sup>-mutant metastatic melanoma responded radiographically to the MEK inhibitor TAK-773. Collectively, these data show that tumor cells harboring BRAF L597 and possibly K601E mutants can be dependent upon ERK signaling and therefore susceptible to treatment with MEK inhibitors. Although prior studies have shown that BRAF L597 mutations are activating (7), to our knowledge, this is the first report on the sensitivity of such mutations to MEK inhibitors in vitro and in patients.

Whether MEK inhibitors will be clinically better than mutant BRAF inhibitors for BRAF L597 and K601E mutants cannot be determined by the in vitro experiments conducted in this study. Cells expressing BRAF<sup>K601E</sup> have been shown to be moderately inhibited by vemurafenib; however, the few patients whose melanomas harbored this mutation did not display responses to another BRAF inhibitor, GSK2118436 (dabrafenib; refs. 11–13). There is 1 patient with a K601E mutation in BRAF that did respond to GSK1120212 (trametinib; ref. 14). Additional in vivo and human studies will need to be conducted to address which class of agents is the most appropriate.

The choice of tumor mutations to be interrogated in the clinic requires a balance among feasibility, cost, and comprehensiveness. Single-mutation testing [i.e., BRAF<sup>V600E</sup> (c.1799T>A)] is relatively cost effective but will clearly overlook other actionable mutations, as shown here. Whole-genome sequencing is comprehensive, but currently prohibitively expensive for routine clinical use. Presently, an intermediate solution involves use of multiplex tests that can interrogate a limited number of known mutations in selected genes that may act as targets for drug therapy. For example, tumors from patients with malignant melanomas at Vanderbilt are routinely screened for recurrent mutations that occur with at least greater than or equal to 1% frequency in the disease [i.e., BRAF (V600E/K/M/R/D), NRAS (G12/13, Q61), KIT (W557, V559, L576, K642, and D816), GNAQ (Q209), and GNA11 (Q209)], using a SNaPshot-based assay (4). Such screening results in 33% of cases having a “pan-negative” status (Supplementary Fig. S11). Non-V600 mutations in BRAF were not originally chosen for examination, as these were observed to occur in the COSMIC database at a frequency of less than 1% (5). The observations that L597 and K601 mutations may occur at a frequency of 4% and 2%, respectively, in “pan-negative” cases and that patients harboring tumors with these mutations may be sensitive to MAPK pathway inhibitors suggest that such tumors should subsequently undergo BRAF exon 15 mutational analyses to exclude the possibility of rare but potentially actionable BRAF mutations.

WGS of the tumor/normal pair also revealed a number of other somatic mutations potentially relevant to melanoma biology (Supplementary Table S8). For example, we validated 3 mutations in the glutamate receptor encoded by GRIN2A (c.3395C>T, p.P1132L; c.3103G>A, p.D1035N; and c.C154T, p.R52X). GRIN2A mutations were recently reported to occur in up to 33% of melanomas (15). The role of this mutation in melanoma is currently unknown. We also identified a tumor-specific noncanonical KRAS mutation (c.466T>C; p.F156L) that has not been previously reported in cancer but has been described as a germline mutation in patients with Noonan or cardio-facio-cutaneous syndromes (16, 17). Interestingly, this mutant protein accumulates in the active conformation, similar to KRAS<sup>G12D</sup>, and expression of an analogous HRAA<sup>G13C</sup> mutation in NIH3T3 cells is transforming (17–19). RAS mutations have been reported to co-occur with BRAF mutations involving codons other than 600 or 601 (20). In addition, we validated other somatic mutations in genes for which importance in melanoma is unspeciﬁed but that are recognized to have important functions in other tumor types, such as APC, BRCA2, NOTCH1, PTEN, and NFI. Future studies will need to be conducted to determine if these alterations are passenger or driver mutations and how they would affect responses to MEK inhibition.

**METHODS**

**DNA Extraction from Patient Samples**

Genomic DNA was extracted from a flash-frozen melanoma thyroid metastasis (90% tumor content) using standard proteinase K digestion and phenol extraction. Genomic DNA from matched patient blood was extracted using the Gentra Puregene Blood Kit (Qiagen). Identity testing was conducted to confirm that tumor and blood genomic DNA originated from the same individual (Applied Biosystems). See Supplementary Methods for patient details. For examining BRAF exon 15 from 49 patient samples, DNA from formalin-fixed paraffin-embedded (FFPE) tissue was extracted using the QIAGEN DNA FFPE Tissue Kit. All patient samples were analyzed with informed consent on an Institutional Review Board (IRB)–approved protocol (IRB 030220 and IRB 100178).

**Whole-Genome Sequencing**

Paired-end sequencing of tumor and matched blood genomic DNA was conducted on an Illumina Genome Analyzer Iix platform. Average coverage was >55.7 and >47.8 for the tumor and matched blood samples, respectively. The 100-bp reads were aligned to the Human Genome (UCSC hg19) using ELAND (Version 2; ref. 21). FastQC (Version 0.9.1; ref. 22) was used to conduct a quality control check of the raw data. See Supplementary Methods for SNP, insertion/deletion, structural variant, and copy number variation detection and validation details.

**Direct Dideoxynucleotide-Based Sequencing**

Somatic SNPs and structural variants were validated by direct sequencing of genomic DNA from the tumor and matched blood (Supplementary Tables S10 and S11). To determine the frequency of BRAF exon 15 mutations, we conducted direct sequencing using genomic DNA extracted from 49 FFPE samples that were pan negative for 43 patient blood was extracted using the Gentra Puregene Blood Kit (Qiagen). Genomic DNA was extracted from a flash-frozen melanoma thyroid metastasis (90% tumor content) using standard proteinase K digestion and phenol extraction. Genomic DNA from matched patient blood was extracted using the Gentra Puregene Blood Kit (Qiagen). Identity testing was conducted to confirm that tumor and blood genomic DNA originated from the same individual (Applied Biosystems). See Supplementary Methods for patient details. For examining BRAF exon 15 from 49 patient samples, DNA from formalin-fixed paraffin-embedded (FFPE) tissue was extracted using the QIAGEN DNA FFPE Tissue Kit. All patient samples were analyzed with informed consent on an Institutional Review Board (IRB)–approved protocol (IRB 030220 and IRB 100178).

**Cloning BRAF Exon 15**

Exon 15 of BRAF was PCR amplified using HotStarTaq Master Mix (Qiagen; Supplementary Table S10). If 2 mutations were detected in exon 15, the PCR products were cloned using TOPO TA Cloning Kit (Invitrogen) to determine if the mutations were in cis or trans. Sequences were analyzed using Mutation Surveyor DNA Variant Analysis Software (SoftGenetics) and manual inspection of the sequence traces.

**Transfections and Drug Treatment**

Wild-type BRAF and BRAF<sup>V600E</sup> plasmids were described previously (23). The BRAF L597R, L597Q, L597S, and K601E mutations were introduced into the wild-type BRAF plasmid using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Direct sequencing of entire cDNAs was conducted to confirm introduction of
the mutation and no other mutations. 293H cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) and 80 ng DNA; cells that were serum starved for 6 hours were treated with vehicle (dimethyl sulfoxide), PLX4032/vemurafenib (ChemoTek), or GSK1120212 (ChemoTek) at 0.01, 0.5, 1, and 5 μmol/L for 2 hours. The 293H cells were passaged in the laboratory for no more than 6 months after receipt. Cells were tested for identity using isozyme and karyotype analysis by Invitrogen.

Western Blotting
Cells were lysed using standard radioimmunoassay assay buffer supplemented with protease and phosphatase inhibitors. Lysates were quantified and subjected to SDS-PAGE and Western blot analysis using the following antibodies: phospho-MEK1/2 (Ser217/221), total-MEK1/2, phospho-ERK1/2 (Thr202/Tyr204), total-ERK1/2, FLAG, and RAF. All antibodies were purchased from Cell Signaling except for FLAG (Sigma-Aldrich) and RAF (Santa Cruz Biotechnology).

Patient Treatment
Subject S8005-514 was treated with oral daily dosing with TAK-733 (Millennium Pharmaceuticals) at 16 mg on days 1 to 21 of 28-day cycles within an ongoing phase I clinical trial (24). Objective response was assessed by CT scans conducted every 2 months according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1; ref. 25). This study is registered with ClinicalTrials.gov (NCT00948467) and full information about this clinical trial will be provided elsewhere when completed.

Disclosure of Potential Conflicts of Interest
K. Dahlman has received an honorarium from Illumina. V. Bozon is an employee of Millennium Pharmaceuticals. N. Rosen has participated on Astra Zeneca, Novartis, and GlaxoSmithKline advisory boards. J. Sosman has participated on advisory boards for GlaxoSmithKline, Roche, and Millennium Pharmaceuticals. A. Ribas has participated on advisory boards for Amgen, Celgene, GlaxoSmithKline, Prometheus, Roche-Genentech, and Millennium Pharmaceuticals. W. Pao was a consultant for MolecularMD, Bristol-Myers Squibb, Clovis, Symphogen, and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K.B. Dahlman, N. Rosen, J.A. Sosman, Z. Zhao, W. Pao.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.B. Dahlman, K. Hutchison, D. Hucks, P. Jia, Z. Su, N. Rosen, C.L. Vnenčak-Jones, J.A. Sosman, A. Ribas, W. Pao.
Writing, review, and/or revision of the manuscript: K.B. Dahlman, J. Xia, K. Hutchison, D. Hucks, V. Bozon, J.A. Glaspys, N. Rosen, C.L. Vnenčak-Jones, J.A. Sosman, A. Ribas, Z. Zhao, W. Pao.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.J. Hicks, J.A. Glaspys, W. Pao.
Performing experiments and providing some of the data: M. Atefi Research study coordinator: S. Branch.
Pathologist, reviewed cases: P. Lyle.
Medical director at Millennium managing the clinical study C20001: V. Bozon.

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**BRAF**<sup>L597</sup> Mutations in Melanoma Are Associated with Sensitivity to MEK Inhibitors

Kimberly Brown Dahlman, Junfeng Xia, Katherine Hutchinson, et al.


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