BRAF L597 mutations in melanoma are associated with sensitivity to MEK inhibitors

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**Running title:** BRAF L597 mutant melanoma

**Keywords:** melanoma, BRAF L597, whole genome sequencing, BRAF inhibitor, MEK inhibitor, TAK-733

**Abbreviations:** COSMIC, Catalogue of Somatic Mutations in Cancer; DP, depth of coverage; FFPE, formalin-fixed paraffin embedded; qual, quality score; RECIST, Response Evaluation Criteria in Solid Tumors; SVs, structural variants; SNPs, single nucleotide polymorphisms; WGS, whole genome sequencing; WT, wildtype.

**Financial support:** VICC Cancer Center Core Grant (CA68485), the TJ Martell Foundation, the Kleberg Foundation, the Seaver Institute, the Wesley Coyle memorial fund, P01CA129243, the Garcia-Corsini family fund, Harry J. Lloyd Charitable Trust (PIP), the NIH/NCI 5K24 CA97588-06 (JS), the American Cancer Society (Mary Hendrickson-Johnson Melanoma Professorship to JS), Stand Up to Cancer (WP), an anonymous foundation, and an anonymous donor. Treatment with TAK-733 in one patient was supported through a clinical trial from Millennium Pharmaceuticals.

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Conflicts of interest: Kimberly Dahlman has received an honorarium from Illumina. Viviana Bozon is an employee of Millennium Pharmaceuticals. Neal Rosen has participated on Astra Zeneca, Novartis, and Glaxo Smith Kline advisory boards. Jeffrey Sosman has participated on advisory boards for Glaxo Smith Kline, Roche, and Millennium Pharmaceuticals. Antoni Ribas has participated on advisory boards for Amgen, Celgene, Glaxo Smith Kline, Promethus, Roche-Genentech, and Millennium Pharmaceuticals. William Pao was a consultant for MolecularMD, Bristol-Myers Squibb, Clovis, Symphogen, and AstraZeneca. The remaining authors state no conflict of interest.

Total: 2,509 word count (excluding references and figure legends); 17 pages; 1 table; 3 figures; 1 supplementary methods; 1 supplementary figure legends; 11 supplementary figures; and 11 supplementary tables.
ABSTRACT

Kinase inhibitors are accepted treatment for metastatic melanomas that harbor specific driver mutations in BRAF or KIT, but only 40-50% of cases are positive. To uncover other potential targetable mutations, we performed whole-genome sequencing of a highly aggressive BRAF (V600) and KIT (W557, V559, L576, K642, D816) wildtype 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 2 (4%) harbored L597 mutations and another 2 involved BRAF D594 and K601 mutations. In vitro signaling induced by L597R/S/Q mutants was suppressed by MEK inhibition. A patient with BRAF L597S mutant metastatic melanoma responded significantly to treatment with the MEK inhibitor, TAK-733. Collectively, these data demonstrate clinical significance to BRAF L597 mutations in melanoma.

SIGNIFICANCE: For the first time, this study demonstrates 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.
INTRODUCTION

Melanoma is a malignant tumor of melanocytes that caused nearly 9000 deaths in the USA 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) (2) or KIT (exons 11 and 13) (3), respectively; however only ~40-50% of cases are positive for these mutations. Currently, clinical algorithms recommend assessing BRAF status in melanomas at only the most common “actionable” mutant site, BRAF V600E (c.1799T>A).

To uncover other potentially targetable mutations, we performed whole-genome sequencing (WGS) of a tumor/normal pair from a patient with a highly aggressive BRAF (V600) and KIT (W557, V559, L576, K642, D816) wildtype (WT) melanoma. Among the many mutations identified, we focused on the biological and clinical significance of a confirmed somatic mutation at the BRAF L597 codon. Our findings have direct therapeutic implications for patients with metastatic melanoma.

RESULTS

Identification of a BRAF L597R mutation by whole genome sequencing

A 75-year old white man presented with an ulcerated right ear melanoma which was widely excised with uninvolved sentinel lymph nodes. Four months later, he developed local recurrence and underwent additional surgery as well as post-operative radiation. This specimen was negative for the BRAF V600E (c.1799T>A) mutation using an allele-specific PCR assay, and negative for KIT exons 9, 11, 13, 17 and 18 mutations 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 performed WGS of DNA from his metastatic thyroid lesion, along with DNA from matched blood (see
**Supplementary Methods** for case description and variation calling). A subset of the single nucleotide polymorphisms (SNPs), insertions, and deletions were validated ([Supplementary Tables S1-S8 and Supplementary Fig. S1-S7](#)). The validated somatic SNP with the combined highest depth of coverage (DP) and quality (qual) scores was BRAF L597R (c.1790T>G) ([Fig. 1](#), [Supplementary Fig. S7](#), and [Supplementary Table S8](#)). The high depth of coverage for this SNP was in part due to concurrent amplification of the BRAF locus on chromosome 7 ([Supplementary Table S5](#) and [Supplementary Fig. S6](#)). Screening of an earlier biopsy confirmed that this mutation was present prior to radiation therapy (data not shown), suggesting that it occurred early in the pathogenesis of the patient’s disease.

**Frequency of BRAF L597 and other exon 15 mutations in “BRAF-wildtype” 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, D816), GNAQ (Q209) and GNA11 (Q209) (4). Two (4%) additional tumors had BRAF L597 mutations (c.1790 T>A, p. L597Q; c.1789_1790 CT>TC, p. L597S). A third tumor harbored a BRAF K601E mutation (c.1801 A>G), while a fourth had a D594N mutation (1780G>A) ([Table 1](#)). The BRAF c.1789_1790 CT>TC 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.

**Signaling induced by BRAF L597 and K601E mutants is suppressed by a MEK inhibitor**

BRAF L597 and K601 are located in the activation segment of the kinase domain and are adjacent to V600. Since V600 mutants are sensitive to specific BRAF and MEK inhibitors, we studied whether signaling induced by L597 and K601 mutants in 293H cells was inhibited by the BRAF mutant
inhibitor, vemurafenib, and the MEK inhibitor, GSK1120212. We chose to study L597R/Q/S and K601E mutations because they have been reported to occur in melanoma in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (5) and we identified these mutations in our “pan-negative” samples (Table 1). We did not study endogenous melanoma cell lines, because to our knowledge, none harbor and are dependent upon an L597 or K601E mutation for survival. We did not further investigate the sensitivity of the D594N mutation because it has been reported that D594 mutations results in an inactive kinase (6). Compared to a vector control, ectopic expression of V600E, L597R/Q/S, and K601E mutants elevated phospho-MEK and -ERK levels (Supplementary Fig. S8), although the L597R/Q mutants did so to a lesser extent, consistent with studies on an analogous L597V mutant (7). Vemurafenib treatment of all of the BRAF mutant-expressing cells led to a decrease in phospho-MEK and -ERK protein levels, while treatment with the MEK inhibitor led to a more dramatic decrease in phospho-ERK signaling (Fig. 2, Supplementary Fig. S8, and Supplementary Fig. S9). These data suggest that patients whose tumors harbor BRAF L597 and K601 mutations could benefit from treatment with MEK inhibitors.

**Objective radiographic response to MEK inhibitor therapy in a patient with a metastatic melanoma with a 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 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 >24 weeks (Fig. 3 and Supplementary Table S9). Follow-up sequencing analysis of DNA from the patient’s tumor revealed a somatic BRAF L597S mutation.
(c.1789_1790CT>TC) mutation (Supplementary Fig. S10). These data validate the notion that BRAF L597S mutated melanomas are sensitive to MEK inhibitors in patients.

DISCUSSION

Inhibitors of mutant BRAF and the MAPK pathway have been demonstrated to improve the overall survival of patients with melanomas that harbor recurrent genetic alterations involving BRAF V600E (c.1799T>A) (2, 9). Here, WGS of a tumor-normal pair revealed that a BRAF “wildtype” tumor harbored an unexpected BRAF L597 mutation occurring in exon 15. Through analysis of additional tumors negative for recurrent mutations in BRAF (V600E/K/M/R/D) as well as NRAS (G12/13, Q61), KIT (W557, V559, L576, K642, D816), GNAQ (Q209) and GNA11 (Q209) (4), we show that BRAF exon 15 mutations not involving the V600 codon are relatively common (4 of 49 samples (8%), including two L597 mutations), consistent with other studies in melanoma (10). We further demonstrate in vitro that signaling induced by ectopic expression of BRAF L597 and K601 mutants is suppressed by MEK and BRAF inhibition. Finally, we report a case of a patient whose BRAF L597S mutant-metastatic melanoma responded radiographically to the MEK inhibitor, TAK-733. 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 in vitro and in patients to MEK inhibitors.

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 performed in this study. Cells expressing BRAF K601E 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®) (11-13). There is one patient with a K601E mutation in BRAF that did
respond to GSK1120212 (Trametinib®) (14). Additional in vivo and human studies will need to be performed 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 V600E (c.1799T>A)), is relatively cost-effective but will clearly overlook other actionable mutations, as demonstrated here. Whole genome sequencing is comprehensive, but currently prohibitively expensive for routine clinical use. Presently, an intermediate solution involves use of multiplex tests which 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, 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 COSMIC 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 three 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 non-canonical 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 G12D, and expression of an analogous HRAS F156L mutation in NIH3T3 cells is transforming (17-19). RAS mutations have been reported to co-occur with BRAF mutations involving codons other than BRAF 600 or 601 (20). In addition, we validated other somatic mutations in genes of which the importance in melanoma are unspecified but are recognized to have important functions in other tumor types, such as APC, BRCA2, NOTCH1, PTEN, and NF1. Future studies will need to be performed 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 (gDNA) was extracted from a flash-frozen melanoma thyroid metastasis (90% tumor content) using standard proteinase K digestion and phenol extraction. gDNA from matched patient blood was extracted using the Gentra Puregene Blood Kit (QIAGEN). Identity testing was performed to confirm that tumor and blood gDNA originated from the same individual (Applied Biosystems). See Supplementary Methods for patient details. For examining BRAF exon 15 from the 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 IRB-approved protocol (IRB #030220 and IRB #100178).

Whole genome sequencing

Paired-end sequencing of tumor and matched blood gDNA was performed on an Illumina Genome Analyzer IIx platform. Average coverage was 55.7x and 47.8x for the tumor and matched blood samples, respectively. The 100 bp reads were aligned to the Human Genome (UCSC hg19) using ELAND.
FastQC (Version 0.9.1) (22) was used to perform a quality control check of the raw data. See Supplementary Methods for single nucleotide polymorphism (SNP), insertion/deletion, structural variant, and copy number variation detection and validation details.

**Direct dideoxynucleotide-based sequencing**

Somatic SNPs and structural variants (SVs) were validated by direct sequencing of gDNA from the tumor and matched blood (Supplementary Table S10 and Supplementary Table S11). To determine the frequency of BRAF exon 15 mutations, we performed direct sequencing using gDNA extracted from 49 FFPE samples that were pan-negative for 43 driver mutations in BRAF, NRAS, KIT, CTNNB1, GNAQ, and GNA11 (4) (see Supplementary Table S10 for BRAF PCR primers). Sequences were analyzed using Mutation Surveyor DNA Variant Analysis Software (SoftGenetics) and manual inspection of the sequence traces.

**Cloning BRAF exon 15**

Exon 15 of BRAF was PCR amplified using HotStarTaq Master Mix (QIAGEN) (Supplementary Table S10). If two mutations were detected in exon 15, the PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) to determine if the mutations were in *cis* or *trans*. Sequences were analyzed using BioEdit v.7.0.5.3.

**Transfections and drug treatment**

WT BRAF and BRAF V600E plasmids were described previously (23). The BRAF L597R, L597Q, L597S, and K601E mutations were introduced into the WT BRAF plasmid using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Direct sequencing of entire cDNAs was performed to confirm introduction of the mutation and no other mutations. 293H cells (Invitrogen) were transfected...
using Lipofectamine 2000 (Invitrogen) and 80 ng DNA; cells serum-starved for 6 h were treated with vehicle (DMSO), PLX4032/vemurafenib (Chemietek), or GSK1120212 (Chemietek) at 0, 0.1 μM, 0.5 μM, 1 μM, 5 μM for 2 h. The 293H cells were passaged in the laboratory for no more than 6 months after receipt. Cells were tested for identity by isozyme and karyotype analysis by Invitrogen.

**Western blotting**

Cells were lysed using standard RIPA 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 BRAF. All antibodies were purchased from Cell Signaling except for anti-FLAG (Sigma-Aldrich) and BRAF (Santa Cruz).

**Patient treatment**

Subject 58005-514 was treated with oral daily dosing with TAK-733 (Millennium Pharmaceuticals Inc., Cambridge, MA) at 16 mg on days 1–21 of 28-day cycles within an ongoing phase 1 clinical trial (24). Objective response was assessed by CT scans performed every 2 months according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) (25). This study is registered with ClinicalTrials.gov (NCT00948467) and full information about this clinical trial will be provided elsewhere when completed.

**ACKNOWLEDGEMENTS**

We would like to thank H. Pan and S. Jelsma for technical assistance with experiments and C. Lovly for critically reviewing the manuscript.
REFERENCES


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

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<td>1</td>
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<td>4</td>
<td>c.1780G&gt;A</td>
<td>p.D594N</td>
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\textsuperscript{a}Samples considered melanoma SNaPshot screen “pan-negative” were those that did not have mutations in \textit{BRAF} (V600), \textit{NRAS} (G12/13, Q61), \textit{KIT} (W557, V559, L576, K642, D816), \textit{CTNNB1} (S37/45), \textit{GNAQ} (Q209) and \textit{GNA11} (Q209).
FIGURE LEGENDS

Figure 1. Detection and validation of the BRAF L597R mutation. A, Alignment of next-generation sequence reads displaying the BRAF L597R mutation compared to 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 presence of a heterozygous BRAF L597 mutation in the tumor but not in the matched blood. Arrows indicate the position of the mutant or WT peaks.

Figure 2. Signaling induced by BRAF L597R and L597S is sensitive to BRAF and MEK inhibitors. Immunoblotting of lysates from 293H cells transfected with plasmids encoding FLAG-BRAF V600E, FLAG-BRAF L597R, or FLAG-BRAF L597S demonstrate that RAF-MEK-ERK pathway signaling can be inhibited by increasing doses (0, 0.1 μM, 0.5 μM, 1 μm, 5 μM) of A, the BRAF inhibitor vemurafenib or B, the MEK inhibitor GSK1120212, 2 h post-inhibitor treatment.

Figure 3. Computed tomography images from a patient with BRAF L597S 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 (left panel) and after 4 cycles of treatment (right panel).
Fig. 1

A

B

Blood

Tumor

1790T

1790T>G

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Research. on June 18, 2017. © 2012 American Association for Cancer Research.
Fig. 2

A

V600E L597R

V600E L597S

Vemurafenib

BRAF

FLAG

pMEK1/2

MEK1/2

pERK1/2

ERK1/2

B

V600E L597R

V600E L597S

GSK1120212

BRAF

FLAG

pMEK1/2

MEK1/2

pERK1/2

ERK1/2
Fig. 3

Baseline
August 2011

Cycle 4
January 2012
BRAF L597 mutations in melanoma are associated with sensitivity to MEK inhibitors

Kimberly B Dahlman, Junfeng Xia, Katie Hutchinson, et al.

Cancer Discovery  Published OnlineFirst July 13, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-12-0097

Supplementary Material  Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2012/07/13/2159-8290.CD-12-0097.DC1

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