BRAF Fusions Define a Distinct Molecular Subset of Melanomas with Potential Sensitivity to MEK Inhibition

Katherine E. Hutchinson, Doron Lipson, Philip J. Stephens, Geoff Otto, Brian D. Lehmann, Pamela L. Lyle, Cindy L. Vnencak-Jones, Jeffrey A. Sosman, and William Pao

Abstract

**Purpose:** Recurrent "driver" mutations at specific loci in BRAF, NRAS, KIT, GNAQ, and GNA11 define clinically relevant molecular subsets of melanoma, but more than 30% are "pan-negative" for these recurrent mutations. We sought to identify additional potential drivers in "pan-negative" melanoma.

**Experimental Design:** Using a targeted next-generation sequencing (NGS) assay (FoundationOne™) and targeted RNA sequencing, we identified a novel PAPSS1-BRAF fusion in a "pan-negative" melanoma. We then analyzed NGS data from 51 additional melanomas genotyped by FoundationOne™, as well as melanoma RNA, whole-genome and whole-exome sequencing data in the Cancer Genome Atlas (TCGA), to determine the potential frequency of BRAF fusions in melanoma. We characterized the signaling properties of confirmed molecular alterations by ectopic expression of engineered cDNAs in 293H cells.

**Results:** Activation of the mitogen-activated protein kinase (MAPK) pathway in cells by ectopic expression of PAPSS1-BRAF was abrogated by mitogen-activated protein kinase kinase (MEK) inhibition but not by BRAF inhibition. NGS data analysis of 51 additional melanomas revealed a second BRAF fusion (TRIM24-BRAF) in a "pan-negative" sample; MAPK signaling induced by TRIM24-BRAF was also MEK inhibitor sensitive. Through mining TCGA skin cutaneous melanoma dataset, we further identified two potential BRAF fusions in another 49 "pan-negative" cases.

**Conclusions:** BRAF fusions define a new molecular subset of melanoma, potentially comprising 4% to 8% of "pan-negative" cases. Their presence may explain an unexpected clinical response to MEK inhibitor therapy or assist in selecting patients for MEK-directed therapy. Clin Cancer Res; 19(24): 6696–702. ©2013 AACR.

Results

**Identification of a PAPSS1-BRAF fusion in a "pan-negative" melanoma**

A 2.2-cm thick (Clark Level V), focally ulcerated, malignant melanoma from the left shoulder of a 27-year-old Caucasian female was screened for common melanoma driver mutations in BRAF, NRAS, KIT, GNAQ, and GNA11 by the Vanderbilt melanoma SNaPshot assay (1) and determined to be "pan-negative." Axillary dissection revealed metastatic involvement of 21 of 25 axillary lymph nodes (initial tumor staging: pT4bN3, stage IIIC), and the area was irradiated. Unfortunately, she developed local recurrence and lung metastasis. She was subsequently treated with experimental and standard immunotherapies. The patient's disease progressed rapidly and she died 9 months later (11 months after initial diagnosis). During the course of treatment, the primary shoulder lesion was sent to Foundation Medicine Inc. (Cambridge, MA) for comprehensive genomic profiling with the FoundationOne™ assay (2). This test involves targeted next-generation sequencing (NGS) of 3,320 exons in 182 cancer-related genes and 37 introns in 14 genes recurrently rearranged in cancer and simultaneously detects single nucleotide variants, insertions, deletions, copy number changes, and select rearrangements (see Supplementary Materials and Methods). Profiling revealed the presence of a large genomic deletion of approximately 350 kb pairs between BRAF intron 8 and an intragenic region of chromosome 7, suggesting a possible gene fusion event. Subsequent targeted RNA sequencing of tumor cDNA identified a novel, in-frame fusion between exon 5 of the sulfurylase kinase PAPSS1 (3'-phosphoadenosine 5'-phosphosulfate synthetase-1) and exon 9 of BRAF generated by a t(4;7)(q24;q34) translocation (Fig. 1).
To determine the effect of the PAPSS1-BRAF fusion on MAPK signaling in cells, we expressed cDNAs encoding FLAG-tagged wild-type (WT) BRAF, mutant BRAF (V600E), WT PAPSS1, or the fusion in 293H cells. Corresponding lysates were probed by immunoblotting with antibodies against phosphorylated and total forms of MEK1/2 and ERK1/2, as well as against PAPSS1, FLAG, and BRAF. Ectopic expression of PAPSS1-BRAF in 293H cells led to increased levels of phosphorylated MEK1/2 and ERK1/2, similar to levels induced by BRAF V600E (Fig. 2A). WT PAPSS1 did not induce MAPK pathway activation (Supplementary Fig. S1). These data confirm that the PAPSS1-BRAF fusion activates the MAPK signaling cascade.

Activation of MAPK signaling by BRAF V600E is sensitive to inhibition by both vemurafenib (a BRAF mutant-specific inhibitor) and trametinib (a MEK inhibitor; ref. 3). To determine if signaling induced by the BRAF fusion was inhibited by these agents, we transfected 293H cells with the V600E or PAPSS1-BRAF cDNAs and treated them with vehicle control or increasing concentrations of vemurafenib or trametinib for 2 hours. Immunoblotting studies with the corresponding lysates showed that BRAF V600E-induced MEK1/2 phosphorylation was effectively reduced by vemurafenib, but MEK1/2 phosphorylation induced by PAPSS1-BRAF was not. Trametinib, however, was effective at reducing ERK1/2 phosphorylation in both V600E- and PAPSS1-BRAF–expressing cells (Fig. 2B). These results suggest that downstream signaling induced by the PAPSS1-BRAF fusion could be abrogated by MEK but not mutant-specific BRAF inhibitors.

Translational Relevance

Through comprehensive molecular tumor profiling, we identified novel BRAF fusions in 2 of 24 patients with melanoma lacking other known recurrent driver mutations in BRAF, NRAS, KIT, GNAQ, and GNA11. Surrogate kinase assays suggest that activated signaling induced by BRAF fusion proteins is sensitive to mitogen-activated kinase kinase (MEK) MAP–ERK kinase inhibition. We also identified two candidate BRAF fusions in another 49 “pan-negative” cases in The Cancer Genome Atlas skin cutaneous melanoma dataset. Thus, BRAF fusions represent a new, potentially clinically relevant target in melanomas possibly treatable with kinase inhibitors.
To determine whether BRAF fusions are recurrent in melanoma, we interrogated 51 additional melanomas from various institutions genotyped with the FoundationOne\textsuperscript{\textregistered}/C212 assay. This cohort was enriched with cases negative for BRAF mutations (V600), likely due to referral bias. Only 8 of 52 (15\%) tumors harbored V600 changes, at least less than half the expected percent in unbiased cohorts (1), and 7 of 52 (15.4\%) harbored non-V600 (D594, L597, K601, etc.) changes. In addition to the PAPSS1-BRAF fusion, we identified another BRAF fusion, this time involving tripartite motif-containing 24 \[TRIM24-BRAF; inv(7)(q32-34q34); Table 1 and Supplementary Fig. S2\]. This tumor was also "pan-negative" for other known "driver" mutations (Fig. 3 and Supplementary Table S1). Similar to PAPSS1-BRAF, ectopic expression of TRIM24-BRAF led to activation of the MAPK pathway which was sensitive to MEK, but not BRAF, inhibition (Supplementary Fig. S3). Thus, in this cohort, BRAF fusions were present in 8\% [2 of 24, 95\% confidence interval (CI), 1.2\%–27.0\%] of "pan-negative" melanomas (Figs. 3 and 4; Supplementary Table S1).

To extend these findings, we also analyzed RNA, whole-genome, and whole-exome sequencing data from an independent dataset available from The Cancer Genome Atlas (TCGA) skin cutaneous melanoma dataset. In 2 of 49 (4.1\%) "pan-negative" cases, we identified sequence reads indicative of potential BRAF fusions, involving CDC27 and TAX1BP1 as 5’ partners (Supplementary Fig. S4). Consistent with these findings, TCGA reverse phase protein array data comparing levels of phosphorylated MEK1/2 in the tumors harboring fusions versus those with BRAF, NRAS, KIT, GNAQ, or GNA11 mutations revealed that the fusion cases harbor phosphorylated MEK1/2 levels similar to, or greater than, levels observed in BRAF or NRAS-mutant melanomas (Supplementary Fig. S5). Collectively, these data suggest that BRAF fusions exist in 4\% to 8\% of "pan-negative" melanomas.

**Discussion**

The classification and treatment of melanomas by known recurrent single-nucleotide driver mutation status in BRAF (V600), NRAS (G12/13, Q61), KIT (W557, V559, L576, K642, D816), GNAQ (Q209), and GNA11 (Q209; ref. 1) has changed standard treatment practice by enabling rationally guided treatment. However, in our experience at Vanderbilt (Nashville, TN), using an established SNaPshot-based assay in the clinic (1), approximately one third of melanomas are still "pan-negative" for these mutations. We recently determined that approximately 8\% of cases negative for these drivers harbor other activating mutations in BRAF exon 15 (D594E/G/H/N/V, L597R/S/Q/V, and K601E/I/N) rather than the better-known V600E/K/M/R/D alterations (4), and we showed in a patient harboring a BRAF L597 mutation that tumor regression could be induced by a MEK inhibitor (4). Here, we have identified

**Table 1. BRAF rearrangements in "pan-negative" melanomas**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detection method</th>
<th>BRAF exon break</th>
<th>5’ partner, exon break</th>
<th>Spanning pairs (n)</th>
<th>Split reads (n)</th>
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<tbody>
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<td>FoundationOne\textsuperscript{\textregistered}</td>
<td>Exon 9</td>
<td>TRIM24, exon 9</td>
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<td>37</td>
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<tr>
<td>FM-Mel30</td>
<td>FoundationOne\textsuperscript{\textregistered}, RNA Kinome Seq</td>
<td>Exon 9</td>
<td>PAPSS1, exon 5</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 2. Signaling induced by PAPSS1-BRAF is more sensitive to MEK inhibition than BRAF inhibition. A, immunoblotting of lysates from 293H cells transfected with vector (empty vector) or plasmids encoding BRAF V600E-FLAG or PAPSS1-BRAF-FLAG demonstrate that the BRAF fusion activates MAPK pathway signaling similarly to BRAF V600E. B, although MAPK pathway signaling induced by expression of BRAF V600E is sensitive to increasing doses (0, 0.1, 0.5, 1, and 5 \(\mu\)mol/L) of the BRAF inhibitor vemurafenib (vem) or the MEK inhibitor trametinib (tra), signaling induced by PAPSS1-BRAF is more sensitive to trametinib than vemurafenib. kDa, kilodalton.
another subset of potentially clinically relevant "pan-negative" melanomas defined by BRAF fusions. Specifically, we found two novel BRAF fusions (PAPSS1-BRAF and TRIM24-BRAF) in 2 of 24 (8%) "pan-negative" melanomas genotyped on an assay that examines the status of 182 cancer-related genes and 37 introns in 14 genes recurrently rearranged in cancer. Ectopic expression of either fusion activates the MAPK pathway (Fig. 2A, Supplementary Fig. S3), and induced signaling is readily diminished by treatment with the MEK inhibitor, trametinib (Fig. 2B, Supplementary Fig. S3). Through mining TCGA skin cutaneous melanoma dataset, we also identified two potential BRAF fusions in another 49 "pan-negative" cases, indicating a frequency of 4.1% in an independent cohort.

PAPSS1 is a bifunctional sulfurylase kinase, with an N-terminal adenosine-5'-phosphosulfate kinase domain and a C-terminal ATP sulfurylase domain (5). Only the adenylylsulfate kinase domain of PAPSS1 remains intact in the PAPSS1-BRAF fusion described herein. TRIM24 is a transcriptional coregulator of nuclear receptors such as the retinoic acid receptor-α (RAR-α; ref. 6) and is known to facilitate ubiquitination of p53 for proteasomal degradation (7). Interestingly, a version of TRIM24-BRAF fusion was identified in the early 1990s in a cDNA library derived from a model of mouse hepatocellular carcinoma (6, 8), but not identified in humans until now. In addition to BRAF, TRIM24 is also fused to the kinase domains of FGFR1 in a myeloproliferative disorder case (8p11 myeloproliferative syndrome; ref. 9) and of RET in a case of papillary thyroid cancer (10). Like PAPSS1-BRAF, we show that expression of TRIM24-BRAF leads to activation of the MAPK pathway, which is sensitive to MEK inhibition (Supplementary Fig. S3).

Although BRAF fusions have been found in other cancers (pilocytic astrocytoma, gastric adenocarcinoma, thyroid cancer, prostate cancer, and melanocytic nevi; Fig. 5;
refs. 11–18), to our knowledge, BRAF fusions have not yet been functionally characterized in malignant melanoma. A BRAF rearrangement was identified previously by break-apart fluorescence in situ hybridization (FISH) in a single malignant melanoma in 2010; however, insufficient sample remained for follow-up analyses that might have identified the fusion partner and allowed for its characterization (18). In addition, an FCHSD1-BRAF fusion was identified in a large congenital melanocytic nevus (LCMN; ref. 13). If left untreated/unresected, LCMN can be a precursor to melanoma, but this is thought to occur in fewer than 5% of LCMN cases (19). Notably, every BRAF fusion characterized to date activates MAPK pathway signaling (11–16, 18) and, when interrogated, had transforming abilities (11, 12, 15, 18). Because PAPSS1-BRAF and TRIM24-BRAF are structured similarly to all other BRAF fusions (Fig. 5), and because we show that both PAPSS1-BRAF and TRIM24-BRAF activate MAPK pathway signaling (Fig. 2 and Supplementary Fig. S3), we expect that these melanoma BRAF fusions will also be transforming. Additional biological studies outside the scope of this article are ongoing.

In protein fusions involving receptor tyrosine kinases (RTK), the 5’ partners usually encode coiled-coil domains, which enable dimerization necessary for kinase activity (20). In the case of BRAF fusions, AKAP9 (11) and TRIM24 are the only 5’ partners that contain coiled-coil domains. BRAF harbors its own small dimerization motif (Arg-Lys-Thr-Arg, RKTR, and amino acids 506–509) spanning exons 12 and 13 (21), which is intact in all currently known BRAF fusions (Fig. 5); therefore, the need for 5’ partners with dimerization ability may not be necessary for BRAF fusion function. In full-length wild-type BRAF, modulation of the RAS-binding domain (RBD) by activated RAS leads to BRAF homo-/heterodimerization and activation (22). This negative regulatory RBD has been replaced by the various 5’ partners in all known BRAF fusions (Fig. 5). Similarly, the recently discovered BRAF V600E splice variants that induce vemurafenib resistance harbor N-terminal exons and

Figure 5. BRAF fusions identified in melanoma and other cancer types. Schematics of wild-type BRAF (top) and all currently known BRAF fusions, including those identified in this study (PAPSS1-BRAF and TRIM24-BRAF). All BRAF fusions break between exons 8 through 11, thus leaving the serine–threonine (S/T) kinase domain of BRAF intact. CRD, cysteine-rich domain; ex, exon; RBD, Ras-binding domain; RKTR, Arg-Lys-Thr-Arg dimerization domain; WT, wild-type.
mutant kinase domain exons, but RBD exons are spliced out, allowing for constitutive dimerization at the RKTR dimerization interface (23). Recently, Sievert and colleagues demonstrated that KIAA1549-BRAF fusion variants can homodimerize with one another; introduction of a dimer interface mutant (R509H) disrupts this interaction (24). Future studies should ascertain the dimerization properties of the various BRAF fusions.

In summary, through NGS analysis of a "pan-negative" melanoma, we identified a novel PAPSS1-BRAF fusion. The fusion protein activates the MAPK pathway, and the induced downstream signaling is sensitive to MEK inhibition. Subsequent analysis of 51 additional melanomas (24 of which were "pan-negative") revealed a second fusion, TRIM24-BRAF, that also activates MEK1/2 and ERK1/2. We also identified two candidate BRAF fusions in TCGA skin cutaneous melanoma dataset. Thus, BRAF fusions may occur in 4% to 8% of the "pan-negative" melanoma population. Coupled with knowledge that the transforming ability of multiple BRAF fusions has already been established (11, 12, 15, 18), we believe that enough evidence exists to raise awareness that BRAF fusions are present in this "pan-negative" population and have implications for MAPK pathway--targeted therapies currently in clinical trials. Their presence may explain an unexpected clinical response to MEK inhibitor therapy or assist in selecting patients for MEK-directed therapy. Collectively, these biochemical and genetic data define an additional molecular subset of melanoma that should be routinely screened for in the clinic, and knowledge about BRAF fusions in melanoma may provide insights into the mechanism of responses to treatment with an expanding list of available kinase inhibitors.

Disclosure of Potential Conflicts of Interest

D. Lipson is employed as Director of Foundation Medicine Inc. and has ownership interest (including patents) in the same. J.S. Ross is employed as Medical Director of Foundation Medicine Inc. and has a commercial research grant from and ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.E. Hutchinson, D. Lipson, P.J. Stephens, B.D. Lehmann, J. Ross, J.A. Pietenpol, J.A. Sosman, J. Puzanov, W. Pao

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Acknowledgments

The authors thank Doug Johnson, Holly Crandall, and Ashlee Greerwich for coordinating patient tissue acquisition and Lynetha Verge for assisting with tissue protocol consent. The authors also thank Sophia Jelsma, Helen Pan, and Jordan Feigertle for technical assistance and/or helpful discussions. Finally, they thank Kimberly B. Dahlman for critically reviewing the manuscript.

Grant Support

Financial support was provided by the James C. Bradford Family Foundation (VICC investigators), the American Cancer Society (Mary Hendrickson-Johnson ACS Melanoma Professorship to J.A. Sosman and ACS Grant #PF-10-226-01-TBG to B.D. Lehmann), the NIH/National Cancer Institute SK24 CA097588-09 (to J.A. Sosman) and NIH CA98131 (Specialized Program of Research Excellence in Breast Cancer to J.A. Pietenpol), and the Joanna M. Nicolay Melanoma Foundation 2013 Research Scholar Award (to K.E. Hutchinson). W. Pao is supported by a Stand Up To Cancer Innovative Research Grant, a Program of the Entertainment Industry Foundation (SU2C-AACR-IRG0409). J.A. Sosman is supported by a Stand Up To Cancer Melanoma Research Alliance Melanoma Dream Team Translational Cancer Research Grant (SU2C-AACR-DT0612).

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Received June 25, 2013; revised October 8, 2013; accepted October 15, 2013; published online December 17, 2013.


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