BRAF Fusion as a Novel Mechanism of Acquired Resistance to Vemurafenib in BRAFV600E Mutant Melanoma

Atul Kulkarni1,2, Husam Al-Hraishawi1, Srilatha Simhadri1,2, Kim M. Hirshfield1,2, Suzie Chen2,3, Sharon Pine1,2, Chandrika Jeyamohan1, Levi Sokol5, Siraj Ali5, Man Lung Teo6, Eileen White1, Lorna Rodriguez-Rodriguez1,7, Janice M. Mehnert1,2,8, and Shridar Ganesan1,2

Abstract

Purpose: Many patients with BRAFV600E mutant melanoma treated with BRAF inhibitors experience a rapid response, but ultimately develop resistance. Insight into the mechanism of resistance is critical for development of more effective treatment strategies.

Experimental Design: Comprehensive genomic profiling of serial biopsies was performed in a patient with a BRAFV600E mutant metastatic melanoma who developed resistance to vemurafenib. An AGAP3-BRAF fusion gene, identified in the vemurafenib-resistant tumor, was expressed in BRAFV600E melanoma cell lines, and its effect on drug sensitivity was evaluated.

Results: Clinical resistance to vemurafenib in a melanoma harboring a BRAFV600E mutation was associated with acquisition of an AGAP3-BRAF fusion gene. Expression of the AGAP3-BRAF fusion in BRAFV600E mutant melanoma cells induced vemurafenib resistance; however, these cells remained relatively sensitive to MEK inhibitors. The patient experienced clinical benefit following treatment with the combination of a BRAF and a MEK inhibitor. Biopsy of the tumor at a later time point, after BRAF and MEK inhibitors had been discontinued, showed loss of the AGAP3-BRAF fusion gene. Mixing experiments suggest that cells harboring both BRAFV600E and AGAP3-BRAF only have a fitness advantage over parental BRAFV600E cells during active treatment with a BRAF inhibitor.

Conclusions: We report acquisition of a BRAF fusion as a novel mechanism of acquired resistance to vemurafenib in a patient with melanoma harboring a BRAFV600E mutation. The acquisition and regression of clones harboring this fusion during the presence and absence of a BRAF inhibitor are consistent with rapidly evolving clonal dynamics in melanoma. Clin Cancer Res; 1–8. ©2017 AACR.

Introduction

BRAFV600E/K mutations are found in approximately half of malignant melanomas (1–3). These activating mutations in the MAPK/ERK pathway drive tumor progression, survival, and metastasis by promoting cell-cycle progression, facilitating escape from apoptosis, and abrogating immune destruction (4). The development of selective BRAF inhibitors, including vemurafenib and dabrafenib, was a landmark in the treatment of melanoma and has led to significant improvements in clinical response rate (RR), progression-free survival (PFS), and overall survival (OS), compared with chemotherapy for patients with BRAF-mutant melanoma (3, 6). Although an initial reduction in tumor volume is observed in the majority of patients with BRAFV600E mutant melanoma treated with BRAF inhibitors, clinical resistance associated with progression of disease develops in most patients and limits the long-term utility of these agents. This has led to the clinical investigation and approval of combination BRAF and MEK inhibitor therapy (dabrafenib plus trametinib; vemurafenib plus combretastatin) that is associated with superior RR, PFS, and OS compared with BRAF inhibitor monotherapy (7–9).

Several mechanisms of acquired resistance to vemurafenib have been identified and these include secondary NRAS mutations (10), mutations in ARAF and CRAF (11), amplification of BRAF (12), activation of other prosurvival signaling pathways, including the phosphoinositide-3-kinase (PI3K) pathway (11), and amplification of upstream receptor tyrosine kinases such as MET (13). To date, over a dozen acquired mechanisms of resistance are described and, often, multiple mechanisms of
resistance are identified in the same patient (12, 14, 15). However, in many cases no clear mechanism of acquired resistance to BRAF-inhibitor therapy is identified, even with exome sequencing, although there often is evidence of reactivation of MAPK signaling. BRAF fusion genes are an alternative mechanism to activate MAPK signaling that was initially described in pediatric gliomas (16) and recently have been found in some melanomas that lack MAPK signaling.

**Translational Relevance**
In this study, we present evidence that resistance to vemurafenib in a BRAFV600E mutant melanoma is associated with selection for a clone with an AGAP3–BRAF fusion gene. These data demonstrate that BRAF fusions may be an underappreciated mechanism of acquired resistance to BRAF inhibitor therapy. The appearance of the AGAP3–BRAF fusion during BRAF inhibitor treatment, and its loss during progression when BRAF inhibitor therapy was discontinued, are consistent with underlying rapid clonal dynamics in response to treatment. These data suggest a rationale for rechallenge with BRAF inhibitors in some clinical settings.

**Materials and Methods**

**Study approval**

Patient case 1 described in this report was enrolled in an Institutional Review Board (IRB)-approved investigational trial for tumor sequencing (protocol2012002075) conducted at the Rutgers Cancer Institute (CINI) in New Brunswick, New Jersey, in accordance with the Belmont Report. This patient provided written informed consent prior to study enrollment. Approval for use of de-identified data for patient case 2, including a waiver of informed consent and a Health Insurance Portability and Accountability Act (HIPAA) waiver of authorization, was obtained from the Western IRB (protocol20152817), in accordance with the U.S. Common Rule, by Foundation Medicine, Inc.

**Tumor sequencing**

Formalin-fixed tumor samples, and DNA extracted from peripheral blood specimens, were analyzed by a comprehensive genomic profiling assay performed on indexed, adaptor-ligated, hybridization-captured libraries targeting all exons of 315 cancer-related genes (FoundationOne) at a commercial CLIA-certified laboratory, Foundation Medicine.

**Construct generation**

The cDNA encoding AGAP3–BRAF fusion gene was synthesized (Life Technologies) and placed in Gateway entry vector. A V5-tagged expression clone (pcDNA 3.2/V5-DEST) was then created by performing an LR recombination reaction using gateway LR cloning II enzyme mix (Invitrogen Life Technologies, cat. No.11791-020) according to the manufacturer’s protocol. The expression of protein was verified by transient transfection of plasmid in 293T cells and Western blot analysis probing with antibody against V5.

**Creation of stable cell line and colony formation assay**

UACC903 melanoma cells with original BRAFV600E mutations were transfected with either empty vector or the V5-tagged AGAP3–BRAF fusion vector. Cells were then selected on Genetin (G418)-containing RPMI media. Expression of fusion protein was verified by Western blot. The colony-forming ability of these cells expressing either the empty vector or the AGAP3–BRAF fusion vector was evaluated by plating 300 cells/well in a 6-well plate. The cells were allowed to grow for 15 days. The colonies were then stained with crystal violet. To evaluate the effect of a BRAF inhibitor (PLX4720) or a MEK inhibitor (AZD6244) on the colony-forming ability of cells expressing the AGAP3–BRAF fusion protein, cells were plated in a 6-well plate as previously described. Cells were treated with different concentrations of drugs (0.25, 50, 75, 100, and 200 nmol/L) and allowed to grow for 15 days supplemented with fresh media every 3 days. The number of colonies was counted after staining with crystal violet. The percentage of survival was plotted compared with untreated cells from the data collected from three independent experiments.

**Cell proliferation assay**

UACC903 cells expressing either empty vector or cells expressing the V5-tagged AGAP3–BRAF fusion protein were treated overnight with BRAF inhibitor (1 μmol/L), MEK inhibitor (500 nmol/L), or the combination of both drugs (500 nmol/L each). Protein extracts were prepared using NETN buffer (Tris pH 8.0, NaCl 150 mmol/L, EDTA 1 mmol/L, NP40 0.5% and protease and phosphatase inhibitors) and resolved by SDS-PAGE. Blots were probed with antibodies against V5 (Bethyl Laboratories), phosphotyrosine-MEK (pMEK) and total MEK, phosphotyrosine-ERK (pERK) and total ERK, and BRAF (Cell Signaling Technology).

**Cell-mixing studies**

To test if the UACC903 cells expressing the AGAP3–BRAF fusion protein have a survival advantage over the parental UACC903 cells harboring the BRAFV600E mutation alone when treated with the BRAF inhibitor, vemurafenib, the UACC903 parental cells harboring BRAFV600E alone were transfected with pCDNA3-GFP empty vector. Green fluorescent protein (GFP)-positive cells were sorted by fluorescence-activated cell sorting (FACS; BD Biosciences Influx high-speed cell sorter) and grown in RPMI medium. UACC903 parental cells (GFP) and cells
expressing the AGAP3–BRAF fusion gene were mixed together in 10:1 ratio (2000:200)/well in a 12-well plate. The mixed population of cells was either untreated or treated with 500 nmol/L of BRAF inhibitor ( vemurafenib) for 7 days. The effect of BRAF inhibition on cell growth was then evaluated by imaging for inhibition on cell growth was then evaluated by imaging for GFP-positive UACC903 parental cells and cells expressing the fusion protein (by differential interference contrast imaging [DIC]) at 10× magnification using a Zeiss Axiovert 200M inverted fluorescence microscope. Twenty images were taken for each treatment. The percentage of GFP-positive (UACC903 parental cells) and non-GFP cells expressing the AGAP3–BRAF fusion protein (by DIC) were quantified before and after treatment with vemurafenib and plotted.

Results

Patient case 1

A 53-year-old man was initially diagnosed 3 years previously with a 0.7-mm-thick melanoma on the anterior chest wall that was treated with wide surgical excision and clear margins. The patient did well for 3 years and then presented with an enlarging left axillary mass. A PET-CT scan showed the presence of a PET-avid 4.4 cm left axillary mass, as well as PET-avid lesions at T4 and the right ilium, consistent with bony metastatic disease. A fine-needle aspiration biopsy of the T4 lesion was performed with pathologic confirmation of metastatic melanoma. A hotspot sequencing assay showed the presence of \( B R A F^{V600E} \) mutation.

The patient was initially treated with the FDA-approved anti-cytotoxic T-lymphocyte associated antigen 4 (CTLA4) antibody therapy, ipilimumab, given the overall low volume of disease; however, he soon developed increasing, intractable back pain. Imaging studies revealed progression of bony metastases, new liver metastases and increasing abdominal and axillary lymphadenopathy (Fig. 1A). Ipilimumab was discontinued; the patient received radiation therapy to the spine with subsequent initiation of vemurafenib therapy. He experienced an initial dramatic response to vemurafenib, with decreasing lymphadenopathy and responses in bony and liver metastases (Fig. 1A and B). A solitary brain lesion found on restaging was treated with gamma knife therapy approximately 2 months following initiation of vemurafenib. The extracranial disease response was maintained for nearly 4 months at which time the axillary mass, as well as lesions in the liver and spleen, began to enlarge (Fig. 1C).

After informed consent, the patient was enrolled in the CINJ Precision Medicine tumor sequencing protocol. The enlarging axillary mass was biopsied while the patient was still receiving vemurafenib, and metastatic melanoma was confirmed on pathologic analysis. This specimen was sent for comprehensive genomic profiling (FoundationOne, Foundation Medicine), which identified both a \( B R A F^{V600E} \) mutation and a novel AGAP3–BRAF fusion gene involving the joining of exons 1 to 9 of AGAP3 in frame to exons 9 to 18 of BRAF. Also present were a \( V H L^{G527C} \) nonsense mutation, \( C D K N 2 A / B \) loss, an alteration in \( S T K 1 1 \) (SK11 splice site 464+1G>C), an \( A R I D 1 A \) truncating mutation (ARID1A*V560*), and a subclonal mutation in \( D N M T 3 A \) (DNMT3A*R736H). (Table 1). To determine if the AGAP3–BRAF fusion was detectable prior to treatment with vemurafenib, a pretreatment biopsy specimen was also sent for sequencing using the same assay. The pretreatment specimen had the \( B R A F^{V600E} \) mutation but no BRAF fusion. Identical alterations in \( V H L \), and \( C D K N 2 A \) loss, were also present in the pretreatment biopsy specimen. Intriguingly, the pretreatment biopsy had focal deletion of \( S T K 1 1 \) but no evidence of the splice-site mutation. The \( D N M T 3 A^{R736H} \) mutation was also present subclonally in the pretreatment specimen (Table 1).

These results were presented at the CINJ Molecular Tumor Board. Following panel discussion, it was recommended that the patient be treated with combination BRAF inhibition and MEK inhibition, based on prior disease progression on ipilimumab therapy and the fact that he was not a candidate for any U.S.-based trials of nivolumab or pembrolizumab available at that time due to his history of brain metastases. The patient was treated with the combination of dabrafenib and trametinib and initially experienced marked clinical improvement with significant reduction of right upper quadrant pain and overall improvement in quality of life. However, the patient experienced increasing right-upper quadrant pain accompanied by an increase in the left axillary mass within 2 months, and evidence of disease progression was observed on imaging.

Dabrafenib and trametinib were then discontinued. The patient was subsequently treated with anti-programmed death-1 (PD-1) antibody therapy (pembrolizumab) when it became available as part of an expanded access protocol. However, he again had worsening disease in the same left axillary tumor, which became painful within several months of initiating pembrolizumab therapy. The patient underwent palliative resection of this tumor and continued on anti–PD-1 therapy. Nevertheless, he soon exhibited clear evidence of disease progression at multiple tumor sites and anti–PD-1 therapy was discontinued. Comprehensive genomic profiling was again performed on the

---

**Figure 1.**

Representative CT images showing left axillary metastasis and liver metastases. **A**, Prior to vemurafenib treatment; **B**, after 2 months of vemurafenib therapy; and **C**, following development of disease progression in the left axilla on vemurafenib.
Table 1. Next-generation DNA sequencing results on serial biopsy specimens

<table>
<thead>
<tr>
<th></th>
<th>Pretreatment biopsy (bone)</th>
<th>Axillary lymph node biopsy (progressing on vemurafenib)</th>
<th>Axillary lymph node biopsy (progressing on immunotherapy, off kinase inhibitors for ≥3 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Computational purity estimate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BRAF</strong>V600E MAF (depth)</td>
<td>40%</td>
<td>57%</td>
<td>65%</td>
</tr>
<tr>
<td><strong>VHL</strong>E52 MAF (depth)</td>
<td>33% (537)</td>
<td>22% (567)</td>
<td>15% (123)</td>
</tr>
<tr>
<td><strong>ARID1A</strong>Y560* (depth)</td>
<td>0% (670)</td>
<td>8% (676)</td>
<td>0% (197)</td>
</tr>
<tr>
<td><strong>DNNMT3A</strong>R736H (depth)</td>
<td>1% (561)</td>
<td>2% (642)</td>
<td>0% (132)</td>
</tr>
<tr>
<td><strong>STK11</strong> splice-site 464+1G-C (depth)</td>
<td>0% (229)</td>
<td>45% (348)</td>
<td>36% (78)</td>
</tr>
<tr>
<td><strong>STK11</strong> copy-number alteration</td>
<td>Loss</td>
<td>Loss</td>
<td>Loss</td>
</tr>
<tr>
<td><strong>CDKN2A/B</strong> copy-number alteration</td>
<td>Loss</td>
<td>Loss</td>
<td>Loss</td>
</tr>
<tr>
<td><strong>AGAP3–BRAF</strong> fusion</td>
<td>Absent (0 supporting reads in manual confirmation)</td>
<td>Present with 23 supporting chimeric read pairs</td>
<td>Absent (0 supporting reads in manual confirmation)</td>
</tr>
<tr>
<td><strong>BRAF</strong> intron 8 average coverage</td>
<td>546.57</td>
<td>568.21</td>
<td>125.46</td>
</tr>
</tbody>
</table>

NOTE: Asterisk denotes stop codon.
Abbreviation: MAF, mutant allele frequency.

resected left axillary lymph node tumor which had progressed in the absence of BRAF- or MEK-targeted therapy. Biopsy results from the resected tumor specimen revealed the presence of the **BRAF**V600E mutation, but no evidence of the **AGAP3–BRAF** rearrangement (Table 1). Of note, because the region of intron 8 of **BRAF** harboring the breakpoint of the gene rearrangement was sequenced to >125× depth in all samples (Table 1), the presence of the fusion should have been detected even if present at low frequencies. **CDKN2A** loss and the **VHL**E52 and **STK11** splice-site mutations seen in prior axillary lymph node biopsy specimen were also present.

Following review of these results at the CINJ Molecular Tumor Board, reinitiation of the combination of dabrafenib and trametinib was recommended. Unfortunately, the patient had progression of brain metastases that required treatment with whole brain irradiation, delaying initiation of this treatment. Therapy with a BRAF inhibitor alone was ultimately initiated, resulting in a mixed response characterized by shrinkage of liver metastases but clear evidence of disease progression at other sites.

**AGAP3–BRAF fusion**

Analysis of the junction sequence of the **AGAP3–BRAF** fusion showed that intron 8 of **BRAF** was rearranged to intron 9 of **AGAP3**, located telomeric to **BRAF** on chromosome 7, to create an in-frame fusion gene that encodes for a protein containing exons 1 to 9 of **AGAP3** and exons 9 to 18 of **BRAF**. This fusion protein includes the full kinase domain of **BRAF** but lacks the Ras-binding domain and the cysteine-rich domain of the **BRAF** gene (18). A similar **AGAP3–BRAF** fusion has been reported in melanoma and colon cancer, but has not been functionally characterized (19). These junction sequences are consistent with a balanced inversion event in chromosome 7 (Fig. 2 A and B).

**AGAP3** (ArfGAP with GTPase domain, ankyrin repeat and PH domain 3) is a gene encoding for a protein with an N-terminal region containing tandem atypical GTPase domains and paired EF Hands (together comprising a Miro domain), a PH domain, a classical ArfGAP domain, and tandem ankyrin repeats (Fig. 2B; ref. 20). The fusion protein includes amino acids 1 to 407 of **AGAP3** fused to amino acids 381 to 766 of **BRAF**. This fusion protein would include the N-terminal Miro domain and part of the PH domain of **AGAP3** fused to the kinase domain of **BRAF** (Fig. 2B).

Functional characterization of **AGAP3–BRAF** fusion protein

The melanoma cell line UACC903 with a known **BRAF**V600E mutation was stably transfected with either vector alone or vector expressing the **AGAP3–BRAF** fusion. Expression of the fusion protein was confirmed by Western blotting (Fig. 2C). Compared with UACC903 cells expressing empty vector, cells expressing the **AGAP3–BRAF** fusion gene had similar basal levels of phosphorylated-MEK, but increased basal levels of phosphorylated-ERK (Fig. 2C). Treatment with a BRAF inhibitor, PLX4720, for 6 hours abolished MEK and ERK phosphorylation in UACC903 parental cells, but not in UACC903 cells expressing the **AGAP3–BRAF** fusion gene. Treatment with a MEK inhibitor, AZD6244, for 6 hours was able to significantly reduce MEK and ERK phosphorylation in both parental and UACC903 cells expressing the **AGAP3–BRAF** fusion gene (Fig. 2C). Colony formation assays demonstrated that cells harboring the **AGAP3–BRAF** fusion, unlike parental UACC903 cells harboring the **BRAF**V600E mutation alone, were relatively resistant to treatment with a BRAF inhibitor. However, the UACC903 parental cells harboring the **BRAF**V600E mutation alone, and the cells also expressing the **AGAP3–BRAF** fusion were similarly sensitive to treatment with a MEK inhibitor (Fig. 2D).

To complement the colony formation assay, a cell proliferation assay was also performed, in this case using the clinically relevant MEK inhibitor, trametinib. In this assay, UACC903 cells harboring both the **BRAF**V600E mutation and the **AGAP3–BRAF** fusion gene were significantly less sensitive to BRAF inhibition than parental UACC903 cells (Fig. 2E). Both the UACC903 parental as well as cells expressing the fusion gene were sensitive to MEK inhibitor (trametinib), however, at higher concentrations (500 and 1,000 nmol/L) the cells harboring both the **BRAF**V600E mutation and the **AGAP3–BRAF** fusion gene were less sensitive to MEK inhibition compared with parental cells (Fig. 2E). Although this relative resistance was seen only at high trametinib concentrations, it suggests that the presence of the BRAF fusion may also induce some partial resistance to MEK inhibition.

Cells expressing the **AGAP3–BRAF** fusion gene have a growth advantage in the presence of BRAF inhibitor

Mixing studies to evaluate the relative growth advantage that the **AGAP3–BRAF** fusion may impart to melanoma cells already harboring a **BRAF**V600E mutation showed that, in the setting of treatment with vehicle alone, around 90% of the cells were the
parental UACC903 cells, retaining the original ratio of mixed cells (Fig. 3A). However, when cells were treated with vemurafenib for 7 days, the percentage of GFP-positive cells decreased dramatically, to <20% of cells. This result demonstrates that, in the absence of a BRAF inhibitor, cells harboring both BRAF<sup>V600E</sup> and the AGAP3–BRAF fusion gene do not have a significant growth advantage. However, treatment with a BRAF inhibitor resulted in a relative dominance of cells harboring the AGAP3–BRAF fusion. These data suggest that cells harboring both BRAF<sup>V600E</sup> mutation and the AGAP3–BRAF fusion gene are only more fit than parental cells harboring the BRAF<sup>V600E</sup> mutation alone when in the presence of BRAF inhibitor treatment.

Patient case 2

In addition to the index case, we report on an additional patient case demonstrating the co-occurrence of a BRAF rearrangement with a BRAF<sup>V600E</sup> mutation in the setting of acquired resistance to vemurafenib and/or MEK inhibitors. Patient case 2 is a 48-year-old man with metastatic melanoma harboring a BRAF<sup>V600E</sup> mutation who developed progressive disease following treatment with the combination of dabrafenib and trametinib. DNA sequencing of a biopsy of a progressive lesion showed the resistant tumor to harbor both a BRAF<sup>V600E</sup> mutation and a CSTF3–BRAF fusion gene that retained the full kinase domain of BRAF.

Figure 2.

Structural and functional characterization of the AGAP3–BRAF fusion. A, Illustration of AGAP3–BRAF rearrangement event resulting from an inversion in chromosome 7. B, Representation of BRAF and AGAP3 genes and key domains. RBD, Ras binding domain; CR, Cysteine-rich domain; Miro, Mitochondrial Rho GTPase domain; PH, Pleckstrin homology domain. C, UACC903 cells harboring BRAF<sup>V600E</sup> expressing either empty vector or V5-tagged AGAP3–BRAF fusion were either untreated or treated with PLX4720 (BRAF inhibitor), AZD6244 (MEK inhibitor), or both agents. Cell extracts were processed for Western blotting and probed with the indicated antibodies. D, Parental UACC903 cells and UACC903 cells with stable expression of V5-AGAP3–BRAF were treated with increasing concentrations of BRAF inhibitor (PLX4720) left panel or MEK inhibitor (AZD6244), right panel and colony formation is plotted. E, Parental UACC903 cells and UACC903 cells harboring the AGAP3–BRAF fusion were treated with increasing concentrations of a BRAF inhibitor (vemurafenib, left) or a MEK inhibitor (trametinib, right), for 4 days and the cell proliferation was measured with Celltiter 96 Aqueous One Solution reagent (Promega), and percent cell proliferation is plotted as an average of three independent experiments.
Discussion

Standard testing for BRAF mutations is usually undertaken by amplicon-based DNA sequencing to identify hotspot BRAF mutations (21). These assays, however, will not detect BRAF fusions, as the coding sequences of individual exons are not altered. Similarly, BRAF fusions are not detectable with standard whole-exome sequencing, as the fusion junctions are located in the introns. One approach to identifying rearrangements is through hybrid capture of “hotspot introns.” High-depth sequencing of select introns not only sensitively detects rearrangements but analysis of the junction sequence can identify the partner genes involved. As intron 8 of BRAF is captured in the FoundationOne assay, it can identify novel rearrangements of BRAF involving this region. Other targeted approaches to identify fusion proteins include target enrichment based RNA-seq analysis, where target enrichment can be accomplished by hybrid capture, single primer-extension technology, or the anchored multiplex polymerase chain reaction (PCR) approach (22, 23).

BRAF fusion proteins were initially reported and characterized in pediatric pilocytic astrocytomas (16). The majority of pilocytic astrocytomas harbor gene fusions that result in an in-frame fusion of the N-terminal exons of KIAA1549 to exons 9 to 18 of BRAF, leading to a fusion protein that contains the intact wild-type kinase domain of BRAF. The N-terminal region of KIAA1549 is thought to mediate constitutive dimerization of the fusion protein and thus activation of BRAF kinase activity. Introduction of a first-generation BRAF inhibitor, such as vemurafenib, causes paradoxical activation of this fusion protein and increased MEK phosphorylation (24, 25). Cells harboring BRAF fusions respond to vemurafenib in a manner similar to cells harboring RAS mutations, through downstream MAPK pathway activation. As such, vemurafenib should be avoided in these settings; however, either MEK inhibitors or second-generation “paradox-breaking” BRAF inhibitors may be effective in the setting of BRAF fusions.

BRAF fusions have been reported as primary driver mutations in a subset of melanomas without a BRAFV600E mutation (26) and have been shown to be associated with resistance to BRAF inhibitors (19, 26). Here, we report an AGAP3–BRAF fusion that was detected in a melanoma with a known BRAFV600E mutation at the time of development of resistance to vemurafenib. The presence of the AGAP3–BRAF fusion was not detected in pretreatment samples, consistent with this fusion protein representing the mechanism of resistance to vemurafenib. Of note, it was expected...
that the AGAP3–BRAF fusion harbors a wild-type kinase domain, as seen in other reported cases of BRAF fusion genes, including the prior report of an AGAP3–BRAF fusion. This was confirmed by laboratory studies that demonstrated that the AGAP3–BRAF fusion induces resistance to vemurafenib in cells with preexisting BRAF
\(^{V600E}\) mutation, as compared with cells lines harboring a BRAF
\(^{V600E}\) mutation. Cells harboring both BRAF
\(^{V600E}\) and AGAP3–BRAF fusion remained sensitive to a MEK inhibitor, suggesting that MEK inhibition can overcome this mechanism of resistance (27, 28). Of note, cells harboring both the BRAF
\(^{V600E}\) mutation and the AGAP3–BRAF fusion did show some partial resistance to trametinib at high doses in proliferation assays (Fig. 2E), suggesting that BRAF fusion genes may also impart some resistance to MEK inhibitors. The case showing development of a CSTF3–BRAF fusion in the setting of acquired resistance to the combination of dabrafenib and trametinib also suggests that some BRAF fusion genes may impart clinical resistance to MEK inhibitors as well. The relatively transient response in the index case also supports this possibility.

These findings suggest that cells harboring both the BRAF
\(^{V600E}\) mutation and the AGAP3–BRAF fusion were present at a very low frequency in the pretreatment setting and could not be detected even at relatively high sequencing depth. Treatment with vemurafenib induced selection pressure that allowed preferential growth of cells harboring the BRAF fusion gene, making them the dominant clone and driving tumor resistance and progression. The resistant tumor also had a mutation in ARID1A that was not detected in the pretreatment biopsy; it is not clear if this mutation plays any role in vemurafenib resistance, especially as the mutant allele frequency was relatively low. Of note, the pretreatment biopsy had evidence of STK11 deletion; however, neither of the two later biopsies showed evidence of STK11 deletion, but instead had STK11 splice mutations that should lead to loss of function. This finding is consistent with clonal heterogeneity in this patient with a large tumor burden, with convergent evolution for STK11 loss in both clones. The presence of identical BRAF and VHL mutations and CDKN2A deletion in all samples is consistent with evolution from a common ancestral clone.

Interestingly, the same tumor site noted to have the AGAP3–BRAF fusion and BRAF
\(^{V600E}\) mutation later progressed after 3 months on anti–PD-1 antibody therapy, during which there was no treatment with either a BRAF or a MEK inhibitor. When this site was excised for palliation, sequencing revealed only the presence of the BRAF
\(^{V600E}\) mutation, and no evidence of AGAP3–BRAF fusion despite the fact that the region of intron 8 of BRAF that harbored the breakpoint was sequenced to greater than 125 × depth. This finding suggests that the clone harboring both the BRAF
\(^{V600E}\) mutation and the AGAP3–BRAF fusion may have been more fit than clones harboring the BRAF
\(^{V600E}\) mutation alone in the setting of ongoing treatment with vemurafenib. However, when selection pressure from BRAF and MEK inhibition was not present, clones harboring BRAF
\(^{V600E}\) alone may have had a fitness advantage, thereby dominating the recurrent mass. This hypothesis is supported by the reported data from mixing studies showing relative outgrowth of cells harboring both BRAF
\(^{V600E}\) and AGAP3–BRAF fusion only in setting of BRAF inhibitor treatment (Fig. 3A).

There may be very rapid clonal dynamics under selection for the BRAF inhibitors in malignant melanoma, and dominant clonal populations may rapidly shift during tumor progression when drugs are discontinued (Fig. 3B). Similar rapid clonal dynamics have been reported in colon cancer, with transient emergence of clones harboring KRAS mutations emerging during development of cetuximab resistance followed by their regression when cetuximab is discontinued (29). Rechallenge with cetuximab at the time point when KRAS-mutant clones were undetectable led to clinical response (29).

Due to the possibility of a mixed response, the biopsy showing the presence of the AGAP3–BRAF fusion was obtained when the patient was still receiving vemurafenib therapy. If vemurafenib had been completely discontinued before the biopsy was performed, this mechanism of resistance may not have been detected, as the clone with BRAF
\(^{V600E}\) alone could have rebounded rapidly. Of note, this patient did experience some clinical benefit, although brief in duration, upon rechallenge with a BRAF inhibitor after progression on immunotherapy. This result suggests that rechallenge with BRAF/MEK inhibitors may be of benefit in the setting of interval progression of kinase inhibitor therapy, as has been reported by others (30–32).

This is the first report of a BRAF rearrangement as the mechanism of acquired resistance to vemurafenib in a melanoma with a preexisting BRAF
\(^{V600E}\) mutation. It is possible that such BRAF rearrangements may represent an underappreciated mechanism for acquired resistance to BRAF inhibitors. It is hard to estimate the frequency of such events, as post-resistance biopsies using assays that can identify fusion genes are not routinely performed. Given the potential for rapid clonal dynamics, the timing of biopsy of progressive disease to look for mechanisms of resistance may be critical, as there may be rapid rebound of parental clones following discontinuation of kinase inhibitor therapy. Serial biopsies, or serial use of circulating cell-free tumor DNA assays, may identify clinical settings where rechallenge with BRAF inhibitors after progression off therapy may be effective.

Disclosure of Potential Conflicts of Interest
S.M. Ali has ownership interest (including patents) in Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: A. Kulkarni, S. Chen, S. Pine, E. White, J.M. Mehnert, S. Ganesan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Kulkarni, H. Al-Hraishawi, S. Simhadri, K.M. Hirshfield, S. Chen, S. Pine, C. Jeyamohan, S. Ali, M.L. Teo, J.M. Mehnert
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Kulkarni, H. Al-Hraishawi, S. Simhadri, S. Chen, S. Pine, J.M. Mehnert, S. Ganesan
Writing, review, and/or revision of the manuscript: A. Kulkarni, K.M. Hirshfield, S. Chen, C. Jeyamohan, S. Ali, M.L. Teo, L. Rodriguez-Rodriguez, J.M. Mehnert, S. Ganesan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ali, L. Rodriguez-Rodriguez
Study supervision: J.M. Mehnert
Other (radiology imaging and interpretation): L. Sokol

Acknowledgments
This research was supported by the Functional Genomics and Biospecimen Repository Shared Resource(s) of the Rutgers Cancer Institute of New Jersey (P30CA072720) and a generous gift to the Genetics Diagnostics to Cancer...
References


Received March 23, 2016, revised April 11, 2017; accepted May 16, 2017, published OnlineFirst May 24, 2017.
BRAF Fusion as a Novel Mechanism of Acquired Resistance to Vemurafenib in BRAFV600E Mutant Melanoma

Atul Kulkarni, Husam Al-Hraishawi, Srilatha Simhadri, et al.

Clin Cancer Res  Published OnlineFirst May 24, 2017.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-0758