A Secondary Mutation in BRAF Confers Resistance to RAF Inhibition in a BRAF\textsuperscript{V600E}-Mutant Brain Tumor

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ABSTRACT  
BRAFV600E hyperactivates ERK and signals as a RAF inhibitor-sensitive monomer. Although RAF inhibitors can produce impressive clinical responses in patients with mutant BRAF tumors, the mechanisms of resistance to these drugs are incompletely characterized. Here, we report a complete response followed by clinical progression in a patient with a BRAFV600E-mutant brain tumor treated with dabrafenib. Whole-exome sequencing revealed a secondary BRAFV617F mutation at progression that was not present in the pretreatment tumor. Expressing BRAFV600E/L514V induces ERK signaling, promotes RAF dimer formation, and is sufficient to confer resistance to dabrafenib. Newer RAF dimer inhibitors and an ERK inhibitor are effective against BRAFV617F-mediated resistance. Collectively, our results validate a novel biochemical mechanism of RAF inhibitor resistance mediated by a secondary mutation, emphasizing that, like driver mutations in cancer, the spectrum of mutations that drive resistance to targeted therapy are heterogeneous and perhaps emerge with a lineage-specific prevalence.

SIGNIFICANCE: In contrast to receptor tyrosine kinases, in which secondary mutations are often responsible for acquired resistance, second-site mutations in BRAF have not been validated in clinically acquired resistance to RAF inhibitors. We demonstrate a secondary mutation in BRAF (V600E/L514V) following progression on dabrafenib and confirm functionally that this mutation is responsible for resistance. Cancer Discov; 8(9); 1130–41. ©2018 AACR.

See related commentary by Romano and Kwong, p. 1064.

INTRODUCTION

Oncogenic activation of the ERK signaling pathway is common in human tumors and occurs by multiple mechanisms, including mutational activation of RAS-GTPases, point mutations and fusions in BRAF, dysregulation of receptor tyrosine kinases, and genetic inactivation of the RAS-GTPase activating protein neurofibromin-1 (NF1). These genetic events are drivers of transformation in approximately one third of all human cancers (1, 2). Consequently, there have been significant efforts to develop small molecules that target ERK signaling. ATP-competitive small-molecule RAF inhibitors have been developed and approved for use in patients with advanced melanoma (3, 4). Although the downstream effects of RAF and MEK inhibitors in BRAFV600E-mutant melanoma cells are biologically similar (5), the ability of first-generation RAF inhibitors to inhibit ERK signaling is restricted to tumors harboring mutant BRAFV600E. In tumors with wild-type (WT) RAFs, RAF inhibitors increase MEK and ERK phosphorylation and thus activate signaling (5–8). This mechanism of so-called paradoxical activation involves RAS-dependent dimerization and transactivation of BRAF molecules (6).

Molecular events that increase RAS activity and genetic alterations that cause BRAFV600E to dimerize in a RAS-independent manner prevent pathway inhibition by vemurafenib and dabrafenib (9–16). BRAF fusions, identified in a subset of pediatric low-grade astrocytomas and other solid tumors, and many non-V600E BRAF mutants, which, despite high BRAF kinase activity, function biochemically as RAF homodimers, demonstrate de novo insensitivity to RAF inhibitors (17–19). Molecular events associated with acquired resistance have mostly emerged from studies of patients with melanoma and colorectal cancer treated with the RAF inhibitor vemurafenib or from laboratory efforts to generate models of acquired resistance.

Clinical responses to single-agent RAF inhibition have varied among cancer types, suggesting lineage-specific differences in signaling that may drive de novo and acquired resistance (14, 20, 21). It is currently unknown whether similar lineage-specific mechanisms of acquired resistance may also exist, especially in cancer types where RAF inhibitors remain under investigation. We therefore hypothesized that acquired resistance may be driven by and specific to the tumor type in which BRAFV600E signals and is inhibited, and set out to genomically characterize the longitudinal pre- and postprogression specimens from a patient with a BRAFV600E-mutant anaplastic ganglioglioma who initially responded to, but then progressed on, treatment with dabrafenib.

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We sought to determine the mechanistic basis for an initial dramatic response and subsequent relapse to dabrafenib therapy in a 15-year-old boy diagnosed with a BRAF V600E-mutant left temporal lobe brain tumor. The patient was originally diagnosed at 33 months of age with a low-grade astrocytoma [World Health Organization (WHO) grade 2]. The patient's tumor recurred nearly 10 years later with malignant progression and was at that time characterized as anaplastic ganglioglioma (WHO grade 3). He was treated with radiation and temozolomide, and then at second recurrence with lomustine and vincristine. Prospective sequencing of the resected third recurrence revealed a BRAF V600E mutation, prompting enrollment in the pediatric phase I/II trial of dabrafenib (NCT01677741). A partial response was evident 8 weeks after the initiation of therapy, ultimately evolving into complete radiographic response. However, progressive disease was evident by 40 weeks, when therapy was discontinued and the progressing tumor was resected (Fig. 1A). Our review of the pathology then revealed an anaplastic ganglioglioma with new areas of rhabdomyosarcomatous transformation (Fig. 1A; Supplementary Fig. S1).

We performed whole-exome and transcriptome sequencing (WES and RNA sequencing) on tumor specimens acquired from the third recurrence prior to trial enrollment (pre-dabrafenib) and at the time of progression on therapy (post-dabrafenib). Our analysis of WES data from the pre-dabrafenib tumor reaffirmed the presence of the BRAF V600E mutation along with clonal, likely functional mutations in SMAD4, ATM,
Acquired Secondary Mutation in BRAF Following Dabrafenib

and other genes, a focal homozygous deletion of CDKN2A/B, and multiple large-scale DNA copy-number alterations. Only modest genomic evolution was evident from WES of the post-dabrafenib tumor, as it possessed a high degree of concordance among the DNA copy-number alterations and somatic mutations present before treatment (Fig. 1B; Supplementary Figs. S2 and S3; Supplementary Table S1). As analysis of the WES data suggested a modest DNA copy-number gain of chromosome 7 harboring BRAF (Supplementary Fig. S2), we performed multicolor FISH on a short-term cell culture [Sloan Kettering, brain-tumor, dabrafenib resistant (SK-BT-DR)] generated from fresh tissue of the progression specimen and sections from the pre- and post-dabrafenib tumor specimens. Subclonal heterogeneity of BRAF copy number existed with distinct cell populations with modest (3–10 copies) and more pronounced (11–16 copies) gains. Nevertheless, FISH confirmed that these BRAF gains (3–9 copies) preexisted dabrafenib treatment (Supplementary Fig. S1).

To determine the basis of treatment resistance, we further investigated the WES and transcriptome data for previously characterized mechanisms of resistance to RAF inhibitor therapy in other cancer types (9–11, 22–24). Although we found no such alteration, we did identify a subclonal BRAF L514V mutation in the post- but not pre-dabrafenib tumor (Fig. 1B). Similar multimodality sequencing of SK-BT-DR confirmed it also possessed the BRAF L514V mutation and faithfully represented the genomics of postprogression disease in this patient (Fig. 1B).

Although second-site mutations are often acquired and mediate resistance in other commonly mutated oncogenic targets of therapy such as EGFR, KIT, NTRK, and others, this has never been previously validated in BRAF-mutant patients who have responded and relapsed on RAF inhibitor therapy. Reasoning that BRAF L514V might represent such a secondary mutation acquired in response to dabrafenib therapy, we sought to determine the impact of this allele on BRAF protein structure. We examined the mutant BRAF protein structure bound to dabrafenib, which indicated that the protein structure. We examined the mutant BRAF protein structure bound to dabrafenib, which indicated that the L514 residue is located within the ATP-binding pocket of the αC-β4-loop of the kinase domain (25) and adjacent to the site of drug binding (4 angstroms; Fig. 1C). Furthermore, the BRAF L514V residue is homologous to residues in other oncogenically activated kinases that are mutated as a mechanism of acquired resistance to targeted therapy (Supplementary Fig. S4; Supplementary Table S2).

The post-dabrafenib tumor retained expression of BRAF V600E, confirmed by targeted mass spectrometry–based genotyping (Sequenom). To determine whether the resistant tumor retained activation of ERK signaling, we assessed ERK phosphorylation using IHC analysis in both the pre- and post-dabrafenib tumors. The posttreatment tumor retained activation of ERK signaling as detected by IHC measurement of phosphorylated ERK (pERK), particularly in areas with astrocytic and sarcomatous morphology (Supplementary Fig. S1).

In order to determine whether a rare BRAF L514V-mutant subclone was present pretreatment but not captured by exome sequencing, we performed droplet digital PCR of BRAF L514V. This confirmed its presence in both the post-dabrafenib tumor and short-term cell culture (SK-BT-DR) at a mutant allele burden consistent with exome sequencing of the tumor tissue, but the mutant allele was not detectable in either the normal blood or pretreatment tumor specimens (sensitivity of 0.016%; Supplementary Table S3). To determine whether BRAF L514V was present in cis with V600E, we randomly screened and sequenced 20 individual bacterial colonies transformed by plasmids carrying BRAF coding sequence amplified from SK-BT-DR cells. Although a subset of colonies was WT BRAF, others BRAF V600E, and 25% BRAF V600E/L514V, there was no evidence that BRAF L514V existed in the absence of V600E (Supplementary Fig. S5). Together, these data suggest that BRAF L514V was an acquired secondary mutation on the V600E-mutant allele.

**BRAF**<sub>V600E/L514V</sub> Reduces Sensitivity to Dabrafenib by Inducing BRAF Dimerization

To assess whether BRAF L514V was sufficient to drive clinical resistance to dabrafenib, we assessed the ability of L514V, alone and in cis with V600E, to activate MEK–ERK signaling compared with BRAF V600E and WT BRAF. We utilized four different cellular contexts: (i) SKBR3 cells in which RAS activation is HER2-dependent and lapatinib treatment can produce low levels of RAS activity to study the effects of transfected BRAF variants without the influence of endogenous RAS activity on RAF dimerization (19); (ii) the BRAF<sup>V600E</sup> brain tumor cell line DBTRG-05MG; (iii) the immortalized fibroblast cell line NIH-3T3 with WT BRAF; and (iv) a BRAF<sup>V600E</sup>-expressing, dabrafenib-sensitive melanoma cell line, A375. In the latter model, we stably transduced doxycycline-inducible constructs of BRAF<sup>V600E</sup> and BRAF<sup>V600E/L514V</sup> to study the expression of the BRAF double mutant in the context of endogenous BRAF<sup>V600E</sup>, which recapitulates the BRAF genetics of our patient’s post-dabrafenib tumor. Both BRAF<sup>V600E</sup> (VE) and BRAF<sup>V600E/L514V</sup> (VELV) significantly elevated MEK and ERK phosphorylation over baseline levels (Fig. 2A–E and G). The ability of dabrafenib to inhibit pERK was reduced in BRAF<sup>V600E/L514V</sup> compared with BRAF<sup>V600E</sup> in all cell types studied here (Fig. 2A–C; Supplementary Fig. S6). BRAF<sup>V600E/L514V</sup>-expressed resistance to dabrafenib was dose-dependent. Expressing higher levels of BRAF<sup>V600E/L514V</sup> resulted in further increased resistance to RAF inhibition, whereas overexpression of BRAF<sup>V600E</sup> alone to similar levels resulted in only minimally reduced sensitivity (Fig. 2D). BRAF<sup>V600E/L514V</sup>-expressing cells were less sensitive to dabrafenib at lower doses than were cells expressing BRAF<sup>V600E</sup> alone, as measured by a shift to the right of the curve of pERK inhibition (Fig. 2E and F). Furthermore, the levels of pERK recovered more rapidly over time in BRAF<sup>V600E/L514V</sup> cells following a single dabrafenib treatment than did cells expressing BRAF<sup>V600E</sup> alone (Fig. 2G; Supplementary Fig. S7). Cell proliferation, as measured by growth in culture over 72 hours, was less sensitive to dabrafenib treatment in BRAF<sup>V600E/L514V</sup> compared with BRAF<sup>V600E</sup> cells. Moreover, the ability of BRAF<sup>V600E/L514V</sup>-expressing cells to form colonies in the presence of dabrafenib was significantly increased compared with that of BRAF<sup>V600E</sup> cells (Fig. 2H and I, IC<sub>50</sub>, IC<sub>75</sub>, and IC<sub>90</sub> provided in Supplementary Fig. S8), consistent with the incomplete pERK inhibition we observed. These data indicate that BRAF<sup>V600E/L514V</sup> is sufficient to cause acquired resistance to dabrafenib.
Figure 2. BRAFV600E/L514V is an activating mutation and is resistant to dabrafenib. A, V5-tagged BRAF mutants (VE or VELV) were expressed in SKBR3 cells for 24 hours, followed by lapatinib treatment (1 μmol/L for 1 hour), and then followed by treatment with DMSO or dabrafenib (200 nmol/L) for another hour. UT, untransfected. B, DBTRG-OSMG cells were transiently transfected with V5-tagged BRAFV600E or BRAFV600E/L514V for 24 hours and treated with DMSO or dabrafenib for 1 hour. C–G, A375 [BRAFV600E melanoma] cells stably transduced with retrovirus carrying doxycycline (dox)-inducible GFP-tagged empty vector, or vector encoding V5-tagged BRAF mutants, were treated with doxycycline (150, 200, 150 ng/mL, respectively, in C and E, 50 or 200 ng/mL in D) for 24 hours, followed by treatment with dabrafenib (100 nmol/L) in C, D, and G, dose range as shown in E) for 1 hour (C–F) or over time course as shown in G, A–E and G. Expression and/or phosphorylation of the indicated proteins were assessed by immunoblot. F, pERK signal intensity was quantitated using densitometric analysis and graphed as a function of dabrafenib dose. H, A375 cells treated with dabrafenib at doses shown were assessed for cell viability and graphed as percentage relative to control. Doxycycline was replenished at 50% every 24 hours. Data, mean ± SEM. I, A375 cells as in C–H were grown in soft agar with the indicated doses of dabrafenib (30 and 100 nmol/L). The y-axis represents the number of colonies, expressed as an average of four fields per well, three wells per condition, relative to DMSO controls, for each cell line [data, mean ± SEM; ****, P < 0.0001, unpaired Student t-test].

We next sought to determine the mechanism by which BRAFL514V mediates resistance to dabrafenib. BRAFV600E signals as a monomer, which is the mechanistic basis for the selective inhibition of ERK output in BRAFV600E-mutant tumors by existing type I RAF inhibitors such as vemurafenib and dabrafenib. Notably, the p61 BRAF splice-forms, which are acquired in BRAFV600E-mutant patients treated with type I RAF inhibitors, non-V600 activating BRAF mutants, and BRAF in-frame deletions (residues L485–P490), confer resistance to these agents by inducing RAF dimer formation (10, 19, 26, 27). We hypothesized that BRAFV600E, acquired in cis with V600E, similarly promotes RAF dimerization as its mechanism of acquired resistance to dabrafenib. To address this question, we cotransfected constructs encoding BRAFV600E and BRAFV600E/L514V with V5 or FLAG tags in SKBR3 cells to detect homodimerization using an immunoprecipitation (IP) assay. As expected, ectopic expression of p61 BRAFV600E (10) exhibited a strong signal for the BRAF homodimer (Fig. 3A). BRAF homodimers were minimal in cells expressing WT BRAF, but ectopic expression of BRAFV600E/L514V promoted a greater degree of BRAF homodimerization than did BRAFV600E alone, regardless of medium or high levels of BRAF-mutant expression and independent of RAS activity (Fig. 3A and B; Supplementary Fig. S9).

We then determined the impact of disrupting dimerization induced by BRAFV600E/L514V. Here, we utilized the BRAFR509H tool mutation, which disrupts BRAF dimerization and reduces BRAF kinase activity (6). We introduced the R509H mutation to WT BRAF, BRAFV600E, BRAFV600E/L514V, and BRAFV600E/L514V. Consistent with our IP results, the activity of BRAFV600E/L514V, but not BRAFV600E alone, was reduced by
Acquired Secondary Mutation in BRAF Following Dabrafenib

**Figure 3.** BRAF<sup>V600E/L514V</sup> causes resistance to dabrafenib by formation of dimers. A, V5-tagged and FLAG-tagged WT BRAF and the indicated BRAF mutants were coexpressed in SKBR3 cells (lapatinib treated 1 hour prior to collection). The binding of V5-tagged BRAF proteins to FLAG-tagged BRAF proteins was determined by immunoprecipitation followed by immunoblotting analysis. B, Untransfected (UT) and transiently transfected SKBR3 cells expressing V5 or FLAG-tagged BRAF mutants with low (L), medium (M), and high (H) expression levels were treated with lapatinib for 1 hour. Homodimerization of BRAF<sup>V600E</sup> and BRAF<sup>V600E/L514V</sup> was evaluated by immunoblotting following immunoprecipitation. Relative FLAG signal (IP/WCL) in medium and high levels of BRAF mutants was determined by densitometry analysis. WCL, whole-cell lysate. Statistical analysis was performed using unpaired Student’s t test in replicate experiments. C and D, V5-tagged BRAF (WT; R509H; VE; V600E/R509H (VEH); VELV; or V600E/L514V/R509H (VELVH)) were expressed in SKBR3 cells for 24 hours, followed by lapatinib treatment (1 μmol/L for 1 hour; C and D), then followed by treatment with DMSO (−) or dabrafenib (dab; +; 200 nmol/L) for 1 hour (D only). Relative pMEK and pERK levels were quantitated in replicate experiments and shown as a bar graph with statistical analysis. n.s., not significant. E, A375 (BRAF<sup>V600E</sup>) melanoma cells stably transduced with retrovirus carrying doxycycline-inducible vector encoding V5-tagged BRAF mutants were treated with doxycycline (150 or 25 μg/mL) for 24 hours, followed by treatment with dabrafenib (dose range as shown) for 1 hour. Expression and/or phosphorylation of the indicated proteins were assessed by immunoblot. pERK signal intensity was quantitated using densitometric analysis, and graphed as a function of dabrafenib dose. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
dimerinsufficiency(Fig.3C;SupplementaryFig.S10A).AlthoughBRAF^{V600E/R509H}doesnotalterpERKsensitivity
todabrafenibcomparedwithBRAF^{V600E},pERKlevelswerealteredagreeterextendincellsexpressing
dimer-competentBRAF^{V600E/L514V/R509H}thanthoseexpressing
dimerization-competentBRAF^{V600E/L514V}(Fig.3DandE;
SupplementaryFig.S10B).To distinguish this mechanism of action from a conventional gatekeeper mutation, we
assessed the BRAF kinase activity of exogenously expressed
BRAF^{V600E} and BRAF^{V600E/L514V} with a kinase-dead MEK1
substrate. Indeed, there was no difference in the ability
dabrafenabinhibitBRAFactivity(Supplementary
Fig.S11).

**MEK Inhibition and RAF Dimer Inhibition Are More Effective against BRAF^{V600E/L514V}, Activated ERK
Signaling**

BecauseBRAF^{V600E/L514V}activates dimer-dependentERK
signaling and is insensitive to type I RAF inhibition, we
sought alternative small-molecule therapeutic strategies to
overcome this acquired resistance. We first assessed the
sensitivity of such cells to MEK inhibition. We treated cells
expressingBRAF^{V600E} orBRAF^{V600E/L514V} with a RAF inhibitor
(RAFi; dabrafenib), a MEK inhibitor (MEKi; trametinib), or
their combination. Wetestedcombinati ontherapyinmulpelcellularcontexts to exclude the intrinsic sensitivity of
BRAF^{V600E}, expressing parental cells to MEK inhibition.
Inhibition of ERK phosphorylation inBRAF^{V600E/L514V}-expressing
cells was more sensitive to MEK inhibition or the combina-
tion of RAF and MEK inhibitors than to RAF inhibition
alone(Fig.4AandB;SupplementaryFig.S12).Trametinib
alone, or combined with dabrafenib, however, did not equally
inhibit cell growth ofBRAF^{V600E} andBRAF^{V600E/L514V}mutants
in short-term drug incubation (72 hours, correspondingIC_{50}
values in SupplementaryFig.S13) or in long-term colony
formation assay (2 weeks; Fig.4CandD;Supplementary
Figs.S13andS14), even though the therapeutic combination
effectively reduced pERK in cells exogenously expressing
the double mutant. These data suggest that although cells
possessingBRAF^{V600E/L514V}-mediatedresistancetodabrafenib
retain ERK dependency, this cannot be overcome completely
by MEK inhibition.
Acquired Secondary Mutation in BRAF Following Dabrafenib

To establish a therapeutic modality that is equipotent against BRAF<sub>V600E/L514V</sub>-mediated resistance, we assessed the efficacy of a novel class of RAF inhibitors shown to inhibit mutant forms of BRAF that promote RAF dimerization (type II oC-IN inhibitors; refs. 10, 28). We therefore tested eight compounds against this mutation including type I oC-OUT/DFG-IN inhibitors (vemurafenib and dabrafenib); type I oC-OUT/DFG-IN paradox breakers (PLX7904 and PLX8394) that suppress mutant BRAF without activating ERK signaling in cells bearing upstream activation (29); type II oC-IN/DFG-OUT RAF dimer inhibitors (LY3009120 and TAK-632; refs. 30, 31); and two new RAF dimer inhibitors (BGB3245 and BGB3290; WO 2014206344 A1/US 9670203 B2; ref. 32). We treated both stably transduced A375s and transiently transfect SKBR3 cells with BRAF<sub>V600E</sub> or BRAF<sub>V600E/L514V</sub> to compare the relative effectiveness of these inhibitors (Fig. 5A and B; Supplementary Fig. S15). Among these compounds, only BGB3245 and BGB3290 inhibited monomeric BRAF<sub>V600E</sub> and dimeric BRAF<sub>V600E/L514V</sub> with similar potency for both ERK signaling inhibition and cell growth. Conversely, vemurafenib, dabrafenib, PLX7904, PLX8394, LY3009120, and TAK-632 showed potent inhibition of only BRAF<sub>V600E</sub> and had reduced activity against BRAF<sub>V600E/L514V</sub> (Fig. 5A–D; Supplementary Figs. S15 and S16). The MEK inhibitor trametinib demonstrated a small differential in potency against BRAF<sub>V600E/L514V</sub>, whereas the ERK1/2 kinase inhibitor SCH772984 (33) was able to elicit equipotent inhibition of BRAF<sub>V600E/L514V</sub> compared with BRAF<sub>V600E</sub>, as demonstrated by inhibition of both pERK (Supplementary Fig. S17) and cell proliferation (Supplementary Fig. S16).

To determine the mechanism of the BGB3245 equipotency we observed for BRAF<sub>V600E</sub> and BRAF<sub>V600E/L514V</sub>, we explored its affinity for both the first and second site of the BRAF dimer. We exploited the fact that type I RAF inhibitors bound to the first site in the BRAF dimer reduces its affinity for the second site, which explains how ERK signaling is much more sensitive to these drugs when monomer- rather than dimer-driven (19). To create a condition that only one site of the RAF dimer is occupied by a RAF inhibitor, we pretreated BRAF<sub>V600E/L514V</sub>-expressing A375s cells with LGX818, a potent type I RAF inhibitor with slow off-rate from the first site and rapid dissociation from the second site in RAF dimers (19). Upon LGX818 washout and incubation with increasing concentrations of dabrafenib or BGB3245, respectively, we found that BGB3245 inhibits the mutant BRAF<sub>V600E</sub> monomer and the second unbound site of the BRAF<sub>V600E/L514V</sub> dimer with similar potency (Supplementary Fig. S18). These data provide a mechanistic basis for the unique property of BGB3245 in inhibiting activated dimerization-dependent forms of mutant BRAF.

DISCUSSION

Mutational activation of BRAF is a frequent oncogenic event and is described in multiple types of cancer (34–38). ATP-competitive inhibitors of RAF kinase have been shown to be extremely effective in the treatment of melanoma with
BRAF<sup>V600E</sup> mutation (4, 39–42), and these unprecedented clinical effects are thought to be due to the unusual mechanism of action of these drugs, which inhibit ERK signaling only in tumors with V600-mutant BRAF. Molecular events that increase intracellular RAS activation or cause RAF to dimerize in a RAS-independent manner cause resistance to these drugs. As such, RTK activation, ras mutation, and aberrantly spliced RAF isoforms have been shown to confer resistance in model systems, whereas the latter two lesions have been identified in tumors from patients with acquired resistance (9, 10, 43). Published studies have not validated second-site mutations in BRAF as a mechanism of acquired resistance to RAF inhibitor treatment in patients.

In contrast to receptor tyrosine kinases such as EGFR and KIT, in which second-site mutations are responsible for a significant proportion of clinical resistance to targeted therapy (44–47), second-site point mutations in BRAF are not thought to contribute to acquired resistance to the RAF inhibitors vemurafenib and dabrafenib (48). An in vitro mutagenesis screen (49) and two case reports of single-patient tumor evolution studies (50, 51) identified BRAF<sup>L514V</sup> as a secondary mutation associated with resistance to vemurafenib. In these studies, however, it was either not confirmed that the second-site mutation was absent from the pretreatment specimen (51), or the mutation was known to be present prior to RAF inhibitor exposure (50). Our study is the first, to our knowledge, to unequivocally demonstrate the emergence of a BRAF secondary mutation in a tumor that progressed after initial response to dabrafenib. Rather than BRAF<sup>L514V</sup> preexisting in a rare cell population prior to therapy, we identified no evidence of BRAF<sup>L514V</sup> in the pretreatment sample to a sensitivity of approximately 0.01%.

WES of pre- and post-dabrafenib-treated tumors demonstrated that a single novel point mutation in BRAF emerged throughout the course of the treatment. Our sequencing data, validated by clonal selection of a short-term cell culture derived from the post-dabrafenib tumor and by FISH, suggest that V600E was clonal in both the pre- and posttreatment specimens, and chromosome 7q encoding BRAF was duplicated, and that V600E was the allele that was gained. Our data further suggest that L514V is subclonal, present in approximately 30% of tumor cells exclusively in the posttreatment specimen, that chromosome 7q encoding BRAF as a mechanism of acquired resistance to RAF inhibitor treatment in patients.

Methods

Sample Collection, WES, RNA Sequencing, and Analysis

The patient was treated on the global phase I/II trial of dabrafenib for pediatric patients (NCT01677741) in accordance with the Declaration of Helsinki. Tumors were collected preenrollment and postremoval from (independent of) the trial. Collection and genomic characterization of these samples was conducted under Institutional Review Board (IRB)-approved Research Authorization and Biospecimen Use protocols (MSKCC IRB #06-107 and #10-130) with written informed consent on file. For WES and analysis, refer to Supplementary Methods. Integrated mutation profiling of actionable cancer targets (MSK-IMPACT) was performed as described previously (54, 55). The WES data have been deposited in the NCBI dbGaP archive under accession number phs001629.v1.p1.

Cell Lines, Antibodies, and Reagents

The SK-BT-DR short-term culture was generated from the patient’s tumor at the time of surgical resection, after progression on dabrafenib was noted. Collection of the patient’s tumor material and all subsequent analysis of this tumor were performed in accordance with IRB-approved protocols for the collection and use of biospecimens (MSKCC IRB #06-107 and IRB #10-130). A375, SKBR3, NIH-3T3, and D87T6-OSMG cells were obtained from the ATCC between 2015 and 2018, and verified by short tandem repeat profile for cell line authentication tested after receipt at Johns Hopkins University Core Facility (Baltimore, MD). All these cell lines were grown in the recommended medium, tested negative for Mycoplasma contamination, and passaged in vitro for fewer than 2 months after resuscitation. Doxycycline-inducible A375 cells were maintained in DMEM with 10% tetracycline-free FBS, 2 mmol/L l-glutamine, 1% penicillin–streptomycin, 50 μg/mL hygromycin, and 0.2 μg/mL puromycin (19).

Antibodies against ERK, pERK, MEK, pMEK, V5, and FLAG were obtained from Cell Signaling Technology, and antibody against BRAF was obtained from Santa Cruz Biotechnology. Vemurafenib, dabrafenib, PLX7904, LY3009120, TAK632, LGX818, trametinib, PD0325901, MEK162, SCH772984, GDC-0994, and lapatinib were purchased from Selleckchem; PLX8394 was obtained from MedichemExpress; BGB3245 and BGB3290 were provided by BeiGene (MTA to N. Rosen); doxycycline was from Sigma Aldrich; puromycin and hygromycin stock solutions were purchased from Invitrogen. Drugs for in vitro studies were dissolved in DMSO to yield 10 or 1 mmol/L stock solutions, and stored at −20°C.

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The patient was treated on the global phase I/II trial of dabrafenib for pediatric patients (NCT01677741) in accordance with the Declaration of Helsinki. Tumors were collected preenrollment and postremoval from (independent of) the trial. Collection and genomic characterization of these samples was conducted under Institutional Review Board (IRB)-approved Research Authorization and Biospecimen Use protocols (MSKCC IRB #06-107 and #10-130) with written informed consent on file. For WES and analysis, refer to Supplementary Methods. Integrated mutation profiling of actionable cancer targets (MSK-IMPACT) was performed as described previously (54, 55). The WES data have been deposited in the NCBI dbGaP archive under accession number phs001629.v1.p1.

Cell Lines, Antibodies, and Reagents

The SK-BT-DR short-term culture was generated from the patient’s tumor at the time of surgical resection, after progression on dabrafenib was noted. Collection of the patient’s tumor material and all subsequent analysis of this tumor were performed in accordance with IRB-approved protocols for the collection and use of biospecimens (MSKCC IRB #06-107 and IRB #10-130). A375, SKBR3, NIH-3T3, and D87T6-OSMG cells were obtained from the ATCC between 2015 and 2018, and verified by short tandem repeat profile for cell line authentication tested after receipt at Johns Hopkins University Core Facility (Baltimore, MD). All these cell lines were grown in the recommended medium, tested negative for Mycoplasma contamination, and passaged in vitro for fewer than 2 months after resuscitation. Doxycycline-inducible A375 cells were maintained in DMEM with 10% tetracycline-free FBS, 2 mmol/L l-glutamine, 1% penicillin–streptomycin, 50 μg/mL hygromycin, and 0.2 μg/mL puromycin (19).

Antibodies against ERK, pERK, MEK, pMEK, V5, and FLAG were obtained from Cell Signaling Technology, and antibody against BRAF was obtained from Santa Cruz Biotechnology. Vemurafenib, dabrafenib, PLX7904, LY3009120, TAK632, LGX818, trametinib, PD0325901, MEK162, SCH772984, GDC-0994, and lapatinib were purchased from Selleckchem; PLX8394 was obtained from MedichemExpress; BGB3245 and BGB3290 were provided by BeiGene (MTA to N. Rosen); doxycycline was from Sigma Aldrich; puromycin and hygromycin stock solutions were purchased from Invitrogen. Drugs for in vitro studies were dissolved in DMSO to yield 10 or 1 mmol/L stock solutions, and stored at −20°C.

1138 | CANCER DISCOVERY SEPTEMBER 2018 www.aacrjournals.org
Acquired Secondary Mutation in BRAF Following Dabrafenib

Immunoblotting

Cells were disrupted on ice in NP40 lysis buffer as described previously (56). Protein concentration was determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with specific primary and secondary antibodies, and detected by chemiluminescence with the ECL detection reagents, Immobilon Western HRP substrate Luminol Reagent (Millipore) and Luminol Enhancer Solution (Thermo Fisher Scientific). The membranes were imaged on the ChemiDoc Touch Imaging System (Bio-Rad) and relative changes in pERK were quantitated by densitometry analysis using ImageJ as a function of concentration or treatment. Response curves were generated using Prism 6.

Plasmids and Transfections

The pcDNA3.1-BRAF/5:FLAG and pcDNA3.1-p61 BRAF V600E:VS/FLAG were constructed as described previously (6). Mutations were introduced using Site-Directed Mutagenesis Kit (Stratagene) and confirmed by Sanger sequencing. Cells were seeded in 6- or 10-cm dishes and transfected the following day using Lipofectamine 2000 (Thermo Fisher Scientific) or NeuroPORTER transfection reagent (Genlantis) according to the manufacturer’s instructions.

Immunoprecipitation and In Vitro Kinase Assay

Cells were collected and lysed on ice in lysis buffer (25 mmol/L Tris-Cl pH 7.4, 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (Cell Signaling Technology), protease inhibitor cocktail (Millipore), and sodium orthovanadate (New England Biolabs). Exogenously expressed VS-tagged BRAF proteins were immunoprecipitated using a VS-specific antibody agarose conjugate (Sigma-Aldrich). Immunoprecipitations were carried out at 4°C overnight, followed by four washes with ice-cold lysis buffer. Pull-down complexes were eluted with VS peptide (Sigma-Aldrich) and assayed by immunoblotting. In vitro kinase assays were performed using VS-tagged BRAF proteins isolated from 293H cells and kinase-dead K97R MEK1 (Millipore) as a substrate in the presence of 200 μmol/L ATP. Kinase assays were conducted at 30°C for 15 minutes. MEK phosphorylation was assayed by immunoblotting with phospho-specific antibodies.

Cell Proliferation Assays

Cells were seeded in 96-well plates at 1,500 cells per well and cultured in medium containing doxycycline (200 ng/mL for A375-VE) and 10,000 cells growing in log phase were mixed with agar (0.33%), with the compound indicated was prepared by serial dilutions and then added to the dishes containing adherent cells. Cells were incubated for 72 hours, and doxycycline was replenished at 50% every 24 hours. Cell growth was quantitated using the Cell Counting Kit-8 (Dojindo). For each condition, four replicates of each concentration were measured. Relative survival in the presence of drugs was normalized to the untreated controls after background subtraction. Graphs were generated using Prism 6 based on the average of four replicates. IC50, IC75, and IC80 values were calculated using Compusyn software.

Soft-Agar Colony Formation

Soft-agar assay was performed as described previously (56). Briefly, 10,000 cells growing in log phase were mixed with agar (0.3%), treated with either DMSO or compounds indicated in the presence of doxycycline, and plated on the bottom layer of 0.5% agar in 6-well plates. Cells were incubated at 37°C for 2 weeks, with 200 μL of media containing 0.5% doxycycline pipetted over the surface every 24 hours. Colonies were then stained with crystal violet (Sigma-Aldrich) for 1 hour and four random fields per chamber were acquired using Nikon Eclipse Ti inverted microscope (Nikon). The measurement was based on three replicates for each condition.

Disclosure of Potential Conflicts of Interest

I.J. Dunkel is a consultant/advisory board member for Apexigen, Bayer, Bristol-Myers Squibb, Celgene, Eisai, Ipsi, and Pfizer. N. Rosen has ownership interest (including stock, patents, etc.) in Beigene and Kura and is a consultant/advisory board member for Chuqai, AstraZeneca, Beigene, and Kura. No potential conflicts of interest were disclosed by the other authors.

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Other (NP who cared for the patient): M. Petriccione

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