Identification of Multiple Mechanisms of Resistance to Vemurafenib in a Patient with BRAFV600E-Mutated Cutaneous Melanoma Successfully Rechallenged after Progression

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Abstract

**Purpose:** To investigate the mechanism(s) of resistance to the RAF-inhibitor vemurafenib, we conducted a comprehensive analysis of the genetic alterations occurring in metastatic lesions from a patient with a BRAFV600E-mutant cutaneous melanoma who, after a first response, underwent subsequent rechallenge with this drug.

**Experimental Design:** We obtained blood and tissue samples from a patient diagnosed with a BRAFV600E-mutant cutaneous melanoma that was treated with vemurafenib and achieved a near-complete response. At progression, he received additional lines of chemo/immunotherapy and was successfully rechallenged with vemurafenib. Exome and RNA sequencing were conducted on a pretreatment tumor and two subcutaneous resistant metastases, one that was present at baseline and previously responded to vemurafenib (PV1) and one that occurred de novo after reintroduction of the drug (PV2). A culture established from PV1 was also analyzed.

**Results:** We identified two NRAS-activating somatic mutations, Q61R and Q61K, affecting two main subpopulations in the metastasis PV1 and a BRAF alternative splicing, involving exons 4–10, in the metastasis PV2. These alterations, known to confer resistance to RAF inhibitors, were tumor-specific, mutually exclusive, and were not detected in pretreatment tumor samples. In addition, the oncogenic PIK3CAH1047R mutation was detected in a subpopulation of PV1, but this mutation did not seem to play a major role in vemurafenib resistance in this metastasis.

**Conclusions:** This work describes the coexistence within the same patient of different molecular mechanisms of resistance to vemurafenib affecting different metastatic sites. These findings have direct implications for the clinical management of BRAF-mutant melanoma.

Cancer Therapy: Clinical

Introduction

Melanoma is widely known to be a molecularly heterogeneous disease; however, only recently have we been able to selectively treat patients on the basis of molecular alterations and assess response to therapy in defined subgroups (1). About half of cutaneous melanomas harbor a mutation of BRAF, primarily V600E, leading to activation of the mitogen-activated protein kinase (MAPK) pathway (2–4). RAF inhibitors such as vemurafenib (Zelboraf) inhibit the MAPK pathway and cell proliferation only in BRAF-mutated tumors and have shown remarkable clinical activity, with responses in approximately half of the patients in phase II and III clinical trials leading to the increased overall survival (5–7). Unfortunately, the majority of responding patients eventually develop disease progression, typically within 5 to 7 months. Several mechanisms for acquired vemurafenib resistance have been described, mainly leading to reactivation of ERK, the downstream effector in the MAPK pathway (reviewed in ref. 8, 9). These mechanisms include activating NRAS mutations, BRAF gene amplification, overexpression of MAP3K8/COT, a kinase that directly activates MAP–ERK kinase (MEK) and extracellular signal–regulated kinase (ERK), and alternative splicing of BRAF mRNA. In the latter mechanism, variant BRAFV600E transcripts lacking exons coding for a protein region encompassing the RAS-binding domain, lead to ERK reactivation via RAS-independent BRAF dimerization (10–13). Activation of receptor tyrosine kinases [e.g., insulin-like growth factor 1 receptor (IGF-1R)]

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Translational Relevance

Mutational activation of BRAF, a cytoplasmic serine/threonine kinase, is the most frequent genetic alteration in melanoma, with approximately 50% of tumors expressing the BRAFV600E oncoprotein. Treatment with vemurafenib induces significant tumor regression and improves survival of patients with advanced BRAF-mutated tumors, highlighting the essential role of oncopgenic BRAF in melanoma biology. Drug-resistant disease is the major cause of patient death. We evaluated by ultra-deep sequencing the mechanism(s) of acquired resistance in two subcutaneous metastases occurring in a patient with a BRAFV600E-mutated cutaneous melanoma, successfully rechallenged after initial progression to vemurafenib, and detected two mutually exclusive mechanisms of resistance to vemurafenib. Our findings have strong clinical relevance and could serve as a proof-of-principle for future prospective studies in a larger cohort of patients.

and platelet-derived growth factor receptor beta (PDGFRβ)] may also cause resistance to vemurafenib by promoting alternative signaling pathways, notably the PI3K/AKT/mTOR pathway (11, 14). Most of these mechanisms have been identified through studies in vitro and confirmed in a few patients.

Although the standard treatment consists of a daily administration of the drug until disease progression, one study has reported a successful rechallenge with vemurafenib in 2 patients (15). In addition, a recent study in a mouse xenograft model suggests that interval dosing of vemurafenib in 2 patients (15). In addition, a recent study in a mouse xenograft model suggests that interval dosing of vemurafenib might delay the emergence of genetic resistance (16). In a xenograft model suggests that interval dosing of vemurafenib may delay the emergency of genetic resistance (16). In this study, we report an in-depth analysis of resistant tumor tissue (see Results). Single-cell cloning was conducted from a stock frozen at passage 0 and clones were used for cytotoxicity experiments at passage 10 to 15. Cells were free of Mycoplasma. An additional RNA sample from a skin metastasis carrying the BRAFV600E mutation from an advanced cutaneous melanoma patient naïve to vemurafenib was used for RNA sequencing.

Exome sequencing

Exome capture and library construction were conducted using Agilent SureSelect Human All Exon v4 Kit using DNA from the samples described earlier. Libraries were sequenced on Illumina HiSeq 2000, generating 100 bp paired-end reads. Data processing is described in detail in Supplementary Methods. We obtained a mean haploid exome target coverage of 356× for Pre, 321× for PV1, 268× for PV2, 94× for N1, and 76× for N2. Somatic variants were validated using Ion Torrent sequencing technology (Life Technologies), obtaining a median target coverage of 3,123× for Pre, 4,902× for PV1, 2,184× for PV2, and 3,535× for N2.

RNA seq

Unstranded paired-end RNA-seq libraries were prepared from samples PV1, PV2, and PV1-cell line with the Illumina TrueSeq RNA Sample Preparation Kit v2. Libraries were sequenced on Illumina HiSeq 2000, generating 83.6 million of purity filtered 100 bp paired-end fragments for PV1, 79.3 million for PV2, and 88.4 million for PV1 cell line. Sequence analysis is detailed in Supplementary Methods.

RT-PCR detection of alternatively spliced BRAF

To confirm the presence of alternatively spliced BRAF, cDNA prepared from total RNA using random hexamers was amplified by PCR and amplified products subjected to Sanger sequencing. Primer sequences and conditions are described in the Supplementary Methods.

In vitro cytotoxicity assays

Sensitivity of cultured melanoma cells to BRAF, MEK, and phosphoinositide 3-kinase (PI3K) inhibitors (PLX3042, AZD6244, CI-1040, and GDC-0941, all from Selleck) was assayed as described earlier (17) using WST-1 reagent (Clontech) to measure cell growth. IC50 values were calculated using GraphPad Prism 5 software.

Results

Patient characteristics

A 41-year-old man was diagnosed in 2008 with a non-ulcerated nodular melanoma of the trunk, Breslow thickness of 6 mm. In July 2009, he was referred to us with a...
recurrent metastatic disease confirmed by biopsy in the left axillary nodes. He was treated with therapeutic lymph node dissection and local radiotherapy. After 10 months, he progressed systemically in the lungs, liver, bone, kidneys, and subcutaneous tissue. The BRAFV600E mutation was detected in the biopsied lymph node, and the patient was randomized to the vemurafenib arm of the BRIM3 study (5). He received vemurafenib 960 mg twice a day beginning in June 2010 and achieved a near-complete response, which lasted 7 months (Supplementary Fig. S1), after which he progressed with the appearance of new lesions in the left kidney, retroperitoneal nodes, and an increase in the size of previously responding lesions. He then received four cycles of ipilimumab followed by two cycles of temozolomide, and one cycle of fotemustine with progression of disease and the appearance of new lesions.

In the absence of effective therapeutic options in this setting, we decided to meet the expectations of the patient and in November 2011 reintroduced vemurafenib at standard dose. At this time, the disease burden was extensive and progressive, involving the brain, lungs, right atrial wall, kidneys, adrenal glands, and several subcutaneous areas, as documented by a computed tomography (CT) scan of the thorax, abdomen, and pelvis (Fig. 1A) and brain MRI (Fig. 1B). After only 2 weeks of treatment, the patient showed a partial response in the brain, lungs, and skin, which was confirmed at 6 weeks (Fig. 1A and B) and lasted 4 months. Some skin metastases showed responses followed by stabilization. Interestingly, one metastasis on the right arm, which responded to initial vemurafenib therapy in June 2010 and eventually reappeared during chemotherapy, showed some shrinkage during reintroduction of the drug. In addition, the patient also experienced the appearance of a new, rapidly progressing lesion involving the subcutaneous tissue of the right arm. This difference in behavior prompted us to collect snap-frozen tissue samples, referred to as PV1 and PV2, respectively (Fig. 2), to investigate the molecular mechanism(s) of escape.

**Mutation profiling by exome sequencing**

To investigate the mechanisms of resistance to vemurafenib, we first conducted whole-exome sequencing using the illumina technology of a pretreatment paraffin-embedded lymph node metastasis (Pre) and of the two subcutaneous snap-frozen metastases of the right arm (PV1 and PV2, Fig. 2). Matched blood cells (blood) and paraffin-embedded normal lymph nodes were used as control germ-line samples. The tumor samples were sequenced at high coverage (mean range, 268×–356×). Focusing on exonic mutations, we identified 107 somatic single-nucleotide variants (SNV) in Pre, 139 SNVs in PV1, and 127 SNVs in PV2, generating a set of 202 different SNVs, 82 of which...
were common to all three samples. The nonsynonymous to synonymous ratios were 1.5, 2.0, and 1.6 for Pre, PV1, and PV2, respectively (1.16 for the mutations common to all three samples and 2.14 for nonshared mutations). The number and spectrum of base substitutions are indicated in Fig. 3. C>T transitions, of which 83% to 86% occurred at TpC and CpC sites (Supplementary Table S1), predominated in the samples, indicating UV light-induced damage. Sample PV1 contained the highest number of substitutions, suggesting a larger divergence from the primary melanoma. Furthermore, while the spectrum of mutations common to all three tumors was further enriched in C>T transitions, the latter represented only 25% of nonshared mutations (Fig. 3). Private mutations (i.e., tumor-sample specific) were predominantly C>A, a type of mutation that can result from oxidative damage. Interestingly, T>C transitions were additionally enriched in the private mutations of PV1, but not PV2 (Fig. 3C). T>C mutations have been found to be overrepresented in mismatch repair-deficient tumors (18), thus an excess of such mutations may result from PV1-specific defects in DNA repair or be related to exposure of the progressing tumor mass to chemotherapy drugs (cfr. Fig. 2). It should be noted that we have previously reported a loss of UV-light signature among the private mutations (compared with common mutations) identified in two metastases removed 12 years apart from another melanoma patient, further supporting the existence of different mutagenic steps in melanoma development (17).

A set of 174 SNVs were checked using the Ion Torrent technology and 155 SNVs (89%) were confirmed. The extremely high-medium coverage obtained at the mutation sites (mean range 2,184 x 4,902 x) allowed us to compute precise allele frequencies of the validated mutations in the three tumor samples (Fig. 4A and Supplementary Table S2). All clonal mutations in Pre were also clonal in PV1 and PV2, indicating that the two metastases arose directly from Pre or a tumor genetically very similar to Pre. More than two thirds of PV1-specific SNVs were present at low frequency (subclonal), indicating higher tumor heterogeneity. These subclonal mutations curiously included the majority of the PV1-private C>T transitions (Fig. 3D), indicating a more recent acquisition of this type of mutations. Interestingly, the frequency of NRAS<sup>V600E</sup> mutation was almost double in PV2 (53.4%) compared with Pre (31.3%) and PV1 (25.1%). Copy number alteration (CNA) plots generated from the exome sequencing data showed that all three samples present a trisomy of chromosome 7q (and of the entire chromosome in PV2; Supplementary Fig. S2). However, the B-allele frequencies of the germline heterozygous single-nucleotide polymorphisms (SNP) located on 7q are anticorrelated in PV1 and PV2 (Fig. 4B). This indicates that the 7q amplifications result from independent events involving different alleles (duplication of the wild-type BRAF allele in PV1 and of the mutated allele in PV2). CNA plots also showed that these tumors present a moderate aneuploidy, with common clonal amplifications of 1q, 6p, and 20p, PV1-specific 5p trisomy and PV2-specific 7p and 15 trisomies (Supplementary Figs. S2 and S3 and Supplementary Table S3). Interestingly, a chromosome 10 deletion (leading to the loss of PTEN, an event frequently associated with BRAF mutations; ref. 19) was present in a subpopulation of Pre but was not observed in PV1 or PV2.

**A resistant subcutaneous metastasis, PV1, harbors two NRAS mutations**

Among the PV1-specific mutations, we found two subclonal NRAS mutations in codon 61, leading to Q61K and Q61R amino acid substitutions, with a frequency of 18.2% and 5.5%, respectively. The two mutations were never found on the same sequencing read and were further enriched in a cell line derived from PV1 (29.0% and 12.1%, respectively, Fig. 4C). Mutant NRAS alleles were not amplified. Taking into consideration a 30% normal tissue contamination, it is estimated that approximately

![Figure 3. Somatic single-nucleotide substitutions of Pre, PV1, and PV2 samples. A and B, the number and spectrum, respectively, of exonic mutations in the three metastasis Pre, PV1, and PV2 identified by whole-exome sequencing using the Illumina Hiseq platform. C, the number and type of mutations common to all the three samples (COM), found in one or two samples only (not common, NOT), or private to individual samples (PREpr, PV1pr, and PV2pr). The majority of C>A in the pool of not common mutations were validated with Ion Torrent sequencing using an enzymatic DNA fragmentation and had frequencies higher than 0.2 (Supplementary Table S2), indicating that they were accumulated by the tumor and were not technical artifacts (35). D, number and type of private mutations further subclassified as clonal (pr-clon) or subclonal (pr-sub) using frequencies obtained by the somatic variant caller MuTect (36) and taking into account normal tissue contamination.](clincancerres.aacrjournals.org/dev/10.1158/1078-0432.CCR-13-0661)
70% of the tumor cells in PV1-harbored mutant NRAS (80% in the PV1-derived culture). NRAS mutations have been shown to confer resistance to vemurafenib (11). Consistent with this, the PV1-derived cell culture was resistant to PLX-4032 but still sensitive to MEK inhibitors [Supplementary Fig. S4]. No NRAS mutations were detected by Ion Torrent sequencing in PV2 or Pre. PV1 also harbored the oncogenic mutation H1047R in PIK3CA, the gene coding for the p110α catalytic subunit (20, 21). This mutation was subclonal in PV1 and its corresponding short-time culture (frequency 11.2% and 13%, respectively) and undetectable in Pre and PV2.

PIK3CA-activating mutations have been implicated in resistance to MEK and RAF inhibitors (22, 23). We therefore explored whether the PIK3CA H1047R mutation could be responsible for vemurafenib resistance in a subpopulation of PV1. Clonal cell lines harboring this mutation (clone 5, 10, and 11, shown in Supplementary Fig. S5 to have heterozygous expression) were isolated from the PV1-derived culture and tested for their sensitivity to RAF, MEK, and PI3K inhibitors. All three PIK3CA H1047R clones were resistant to PLX4032 (IC50 range 9–58 μmol/L; Fig. 5A), similar to NRAS-mutated clones assayed in parallel. All cultures tested were only moderately sensitive to GDC-0941 (IC50 range, 0.6–2.4 μmol/L), a highly potent PI3K inhibitor that is active against both wild-type and oncogenic p110 variants, including PIK3CA H1047R (24), suggesting some activation of the PI3 kinase pathway. Combined GDC-0941 and PLX4032 treatment only slightly improved growth inhibition in two of the three PIK3CA-mutated clones (Fig. 5B). Similar results were obtained by cotreatment of PLX4032 with another PI3K inhibitor, LY294002 (not shown). Like the parental uncloned culture, PIK3CA and NRAS-mutated clones were sensitive to the MEK inhibitor AZD6244, with IC50 values below 1 μmol/L, although individual PIK3CA clones showed up to 10-fold difference in sensitivity (Fig. 5A), indicating that growth of these cells is still strongly dependent on MAPK pathway activation. Finally, combinations of AZD6244 with PLX4032 or GDC-0941 did not show a synergistic effect [Supplementary Fig. S6].

Gene expression profiling by RNA sequencing reveals an aberrantly spliced BRAF transcript in the resistant subcutaneous metastasis PV2

Because some of the mechanisms responsible for resistance to vemurafenib are observed at the transcriptional or posttranscriptional level, we additionally conducted poly-A+ RNA sequencing (RNA-seq) on PV1, PV2, and the PV1-derived cell line. In PV2 only, we observed an aberrantly spliced BRAF transcript that accounted for approximately 50% of all BRAF transcripts (Fig. 6A). This splice variant

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**Figure 4.** Characterization of somatic mutations. A, mutant allele frequencies of validated mutations in Pre, PV1, and PV2 samples. The points on or near the axis were classified as Pre, PV1, and PV2 specific. The two clusters and outliers are clonal mutations and were defined by fitting a three-component mixture of trivariate Gaussian distributions on the other points using the expectation maximization algorithm implemented in the Statistics Toolbox of MATLAB. Mutations in cluster 2 lay on the minor allele of trisomic chromosomes 1q, 6p, and 20p. Most of the outliers lay in regions with CNA. B, compares the B-allele frequencies (BAF) of germline heterozygous SNPs located on chromosome 7q of samples PV1 and PV2. The 7q amplification involves different haplotypes. The canonical BRAF mutation V600E is indicated in red. Its location just outside the main cluster is due to 20% to 30% contamination with normal tissue. C, sampling of 100 reads that map to NRAS Q61 codon in PV1 and PV1-derived cell line. The two NRAS mutations Q61K (C→A) and Q61R (A→G) have different frequencies and are mutually exclusive on sequencing reads. Base substitutions are indicated in red for A, blue for G, and green for T. Bases identical to hg19 are indicated in gray.
contains an inframe deletion of exons 4–10 and is one of several aberrantly spliced BRAF forms previously reported as an escape mechanism to BRAF inhibitors (12). These variants lack the RAS-binding domain and can activate ERK in the presence of the inhibitor. The variant seems specific to PV2 as was not detected in PV1 or Pre, as confirmed by PCR (Fig. 6B). Sequencing of the variant product showed that aberrant splicing affects the BRAF V600E transcript (Supplementary Fig. S7). PV1 and PV2 gene expression levels were then compared to identify additional potential tumor-specific mechanisms of escape, focusing on genes belonging to the tyrosine kinase and G protein coupled receptor (GPCR) families, RAS/MAPK and AKT/mTOR signaling pathways (Supplementary Fig. S8 and Supplementary Table S4). HGF, ME1, and MAP3K8/COT genes, whose overexpression can drive resistance to vemurafenib (10, 25, 26), showed higher expression in either PV1 or PV2, although were not within the top 10% most differentially expressed genes. As RNAseq could not be conducted on Pre due to the lack of adequate RNA material, we compared PV1 and PV2 expression profiles with those obtained from a BRAF V600E-unrelated metastasis from a patient naïve to anti-RAF/MEK inhibitors. These comparisons showed similarly high levels of expression for the above genes. Of note, the GPCRs GRM1 and EDNRB, which can activate the AKT pathway (27, 28), were overexpressed in PV2.

Discussion

We have described the occurrence of different mechanisms of resistance to vemurafenib, namely activating NRAS mutations and alternative BRAF splicing, that coexist in a patient that was successfully rechallenged with vemurafenib and eventually progressed. Although both mechanisms of acquired resistance have been previously described (11, 12), they have been so far reported to occur as mutually exclusive mechanisms among patients (12). A few reports have provided evidence that different mechanisms for acquired resistance to RAF inhibition can be present within the same patient. For example, Shi and colleagues analyzed two or more resistant metastases from a few patients and identified a prominent mechanism (i.e., NRAS mutation) in one but not the other tumor sample(s) (13). Similarly, Wilmott and colleagues clearly showed the existence of two phenotypically different nodules within a resistant metastasis, only one of which contained mutated NRAS (29). In either study, other mechanism(s) were not detected. Here, we clearly identify multiple resistance mechanisms to BRAF inhibition in different metastases arising in the same patient, at the same anatomic location and within the same tissue (subcutaneous metastases).

Remarkably, we identified two NRAS-activating mutations affecting codon Q61 in a lesion (PV1) that was already
an estimated 30% of cells in PV1 was a potential vemurafenib-resistance candidate that we further investigated. PIK3CA mutations, of which PIK3CAH1047R is the most frequent, are common in some cancers such as breast and colon carcinomas, but not in melanoma (30, 31). PIK3CAH1047R-mutant protein has been shown to activate the AKT pathway and to contribute to MEK inhibitor resistance (22). Results of cytotoxicity assays using PI3K, MEK, and RAF inhibitors, alone or in combinations, on PIK3CAH1047R-mutated cell clones isolated from PV1, did not suggest a driver role for this mutation in vemurafenib resistance. Supporting these conclusions, some clones isolated from the PV1 culture contained both PIK3CAH1047R and NRAS mutations (unpublished observation), suggesting a different role for mutant PIK3CA protein. Nevertheless, the PI3K pathway seemed to be important in both PIK3CA and NRAS-mutated cells, as shown by the partial effect of GDC-0941 on cell growth and a possible contribution of PIK3CAH1047R to vemurafenib resistance deserves a more thorough investigation. In addition, overexpression of genes such as the receptor tyrosine kinase/ligand couple MET/HGF and the GPCRs GRM1 and ENDR8, detected by RNA sequencing, may also play a role.

Figure 6. Detection of alternatively spliced BRAF transcripts. A, BRAF splice junctions found by RNA-seq in samples PV1, PV2, and in the cell line derived from PV1. BRAF exons and introns are indicated at the top. Splice junctions are represented by an arc from the beginning to the end of the junction. The thickness of the arc is proportional to the number of reads covering the junction. The canonical junctions are represented below and the alternative junctions are represented above the line. Exons 4–10 are aberrantly spliced-out in sample PV2. B, the results of reverse transcriptase PCR (RT-PCR) conducted with primers located in exon 3 and 11 (top) on the indicated samples. Alternatively spliced BRAF (97 bp product) is preferentially amplified over the longer (907 bp) canonical form in PV2, but is undetectable in PV1 and Pre samples. Bottom, amplification of a cDNA fragment (exon 15) similar in size to the alternatively spliced BRAF product as control for RNA quality for the FFPE sample (Pre). PCR was conducted for 35 or 40 cycles (left and right, respectively). Lack of amplification in RT− samples shows specificity for cDNA.

other mechanisms, in addition to NRAS mutations and alternative BRAF splicing, may have further contributed to the acquired resistance in our patient. Indeed, NRAS mutations were estimated to account for resistance in only up to 30% of tumor cells in PV1. Among the identified somatic mutations, the PIK3CAH1047R mutation detected in an estimated 30% of cells in PV1 was a potential vemurafenib-resistance candidate that we further investigated. PIK3CA mutations, of which PIK3CAH1047R is the most frequent, are common in some cancers such as breast and colon carcinomas, but not in melanoma (30, 31). PIK3CAH1047R-mutant protein has been shown to activate the AKT pathway and to contribute to MEK inhibitor resistance (22). Results of cytotoxicity assays using PI3K, MEK, and RAF inhibitors, alone or in combinations, on PIK3CAH1047R-mutated cell clones isolated from PV1, did not suggest a driver role for this mutation in vemurafenib resistance. Supporting these conclusions, some clones isolated from the PV1 culture contained both PIK3CAH1047R and NRAS mutations (unpublished observation), suggesting a different role for mutant PIK3CA protein. Nevertheless, the PI3K pathway seemed to be important in both PIK3CA and NRAS-mutated cells, as shown by the partial effect of GDC-0941 on cell growth and a possible contribution of PIK3CAH1047R to vemurafenib resistance deserves a more thorough investigation. In addition, overexpression of genes such as the receptor tyrosine kinase/ligand couple MET/HGF and the GPCRs GRM1 and ENDR8, detected by RNA sequencing, may also play a role.

The clinical complete regression of PV1 (and other metastases) before regrowth and successful vemurafenib rechallenge lends support to a state of temporary resistance in microscopic tumor deposits, possibly in conjunction with epigenetic changes or particular microenvironment conditions. In this regard, a small subpopulation of slow-proliferating, potentially drug-resistant, melanoma cells that can give rise to a highly proliferative progeny and is associated with JARID1B expression has been described earlier (32). Moreover, a reversible drug-tolerant phenotype has been described in non–small cell lung carcinoma cells treated with tyrosine kinase inhibitors (33).

On the basis of current knowledge and clinical practice, vemurafenib is stopped at tumor progression, as mechanisms of resistance may come into play limiting its efficacy. Our results clearly show that the molecular escape mechanisms can be both multiple and mutually exclusive. This has important clinical implications: first, local treatment of isolated progressing lesions and continuation of vemurafenib could be supported by the fact that the resistance mechanisms are not always shared. This approach is currently being tested in clinical trials (34) with preliminary results that seem to support our hypothesis. Second, the presence of more than one mechanism of resistance in the same patient strongly argues that single biopsy analysis at progression might not reflect the molecular complexity of tumor progression, and therefore might not be sufficient to guide selection of optimal second-line therapy. Finally, the patient in this study is the third one reported to respond to RAF-inhibitor rechallenge (15). Although this is a very small number of patients and the individual drug treatments differed (our patient was treated twice with vemurafenib; whereas in Neys and colleagues, the patients first received combined dabrafenib/trametinib and were later rechallenged either with the same combination or dabrafenib alone), the encouraging
clinical results support the exploration of alternative treatment schedules with RAF inhibitors.

Disclosure of Potential Conflicts of Interest

O.M. is a consultant/advisory board member of Roche Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

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