RESEARCH BRIEF

MAP Kinase Pathway Alterations in *BRAF*-Mutant Melanoma Patients with Acquired Resistance to Combined RAF/MEK Inhibition

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ABSTRACT

Treatment of *BRAF*-mutant melanoma with combined dabrafenib and trametinib, which target RAF and the downstream MAP–ERK kinase (MEK)1 and MEK2 kinases, respectively, improves progression-free survival and response rates compared with dabrafenib monotherapy. Mechanisms of clinical resistance to combined RAF/MEK inhibition are unknown. We performed whole-exome sequencing (WES) and whole-transcriptome sequencing (RNA-seq) on pretreatment and drug-resistant tumors from five patients with acquired resistance to dabrafenib/trametinib. In three of these patients, we identified additional mitogen-activated protein kinase (MAPK) pathway alterations in the resistant tumor that were not detected in the pretreatment tumor, including a novel activating mutation in MEK2 (*MEK2*^{Q60P}). *MEK2*^{Q60P} conferred resistance to combined RAF/MEK inhibition *in vitro*, but remained sensitive to inhibition of the downstream kinase extracellular signal–regulated kinase (ERK). The continued MAPK signaling-based resistance identified in these patients suggests that alternative dosing of current agents, more potent RAF/MEK inhibitors, and/or inhibition of the downstream kinase ERK may be needed for durable control of *BRAF*-mutant melanoma.

SIGNIFICANCE: This study represents an initial clinical genomic study of acquired resistance to combined RAF/MEK inhibition in *BRAF*-mutant melanoma, using WES and RNA-seq. The presence of diverse resistance mechanisms suggests that serial biopsies and genomic/molecular profiling at the time of resistance may ultimately improve the care of patients with resistant *BRAF*-mutant melanoma by specifying tailored targeted combinations to overcome specific resistance mechanisms. *Cancer Discov; 4(1); 61–8. ©2013 AACR.*

See related commentary by Solit and Rosen, p. 27.

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INTRODUCTION

Targeted agents that inhibit key effector kinases of the mitogen-activated protein kinase (MAPK) signaling cascade, including BRAF (vemurafenib or dabrafenib) and MAP–ERK kinase (MEK)1 and MEK2 (tremetinib) have improved progression-free survival and overall survival when used as monotherapy in *BRAF*-mutant melanoma (1–4). However, the majority of patients experience disease progression within 6 to 7 months. Many clinical mechanisms of resistance to monotherapy identified in *BRAF*-mutant melanoma to date result in reactivation of MEK/ERK signaling (5–11). Accordingly, recent therapeutic efforts have focused on increased MAPK inhibition through combined targeting of BRAF and MEK. In a phase I/II trial of combined dabrafenib and tremetinib, this combination increased progression-free survival, objective response, and duration of response as compared with dabrafenib monotherapy (12). Nonetheless, resistance still developed in most patients after an average of 9.4 months. The mechanisms of resistance to combined RAF/MEK inhibition remain poorly understood.

To begin to investigate clinical mechanisms of resistance to combined RAF/MEK inhibition, we performed whole-exome sequencing (WES) and whole-transcriptome sequencing (RNA-seq) on tumor samples obtained from 5 patients with acquired resistance to dabrafenib/trametinib. Here, we describe putative resistance mechanisms to combined RAF/MEK inhibition identified in these patients.

CASE SERIES

Five patients with metastatic *BRAF*-mutant melanoma were selected from a phase I/II study of first-line dabrafenib and trametinib (12). All 5 patients experienced a clinical benefit—defined as complete response (CR), partial response (PR), or stable disease (SD) for at least 6 months as determined by Response Evaluation Criteria In Solid Tumors (RECIST; ref. 13)—before developing progressive disease. Biopsies were obtained before treatment with dabrafenib/trametinib and at the time of disease progression. Patient characteristics are summarized in Table 1 and the clinical histories are detailed in the Supplementary Data.

RESULTS

Although combined RAF/MEK inhibition is predicted to avoid several known mechanisms of resistance to RAF inhibitor monotherapy through enhanced suppression of MAPK signaling, 3 of 5 cases examined nonetheless harbored apparent resistance mechanisms that engage MAPK effectors. Results are summarized in Table 1. Below, we describe specific resistance drivers identified in each patient.

An Acquired *MEK2*^{Q60P} Mutation Confers Resistance to RAF/MEK Inhibition

WES of the resistant tumor from patient 1 revealed a mutation in *MEK2* (*MAP2K2*), a downstream kinase from BRAF in the MAPK pathway and the target of trametinib (Fig. 1A, left). This mutation was not detected in the pretreatment tumor despite robust sequence coverage of this locus (224-fold; Fig. 1A and B; Supplementary Tables S1–S3). RNA-seq data demonstrated that this mutation, *MEK2*^{Q60P}, was expressed in the resistant tumor but not in the pretreatment tumor (Fig. 1A, right). *MEK2* mutations have not previously been identified in patients with acquired resistance to RAF or MEK inhibitors, although similar mutations were found to confer resistance to single-agent RAF inhibitors in a companion study (14). *MEK2*^{Q60P} is homologous to *MEK1*^{Q56P}, which confers resistance to monotherapy with RAF or MEK inhibitors *in vitro* (5) and in post-progression tumor samples from patients with acquired resistance to vemurafenib (11).

Table 1. Clinical characteristics and MAPK pathway resistance mechanisms in patients with acquired resistance to dabrafenib/trametinib

	Gender	Age	Prior systemic therapy for metastatic melanoma	Dabrafenib dosing Trametinib dosing		Best response	Duration of response, mo	MAPK pathway candidate resistance mechanisms
				Start dose	End dose			
Patient 1	M	72	None	150 mg BID 2 mg daily	150 mg BID 2 mg daily	PR (–64%)	3	MEK2^{Q60P}
Patient 2	M	48	None	150 mg BID 2 mg daily	100 mg BID 2 mg daily	PR (–42%)	3	BRAF splice isoform
Patient 3	M	42	None	150 mg BID 1 mg daily	150 mg BID 1 mg daily	SD (–19.5%)	11	BRAF amplification
Patient 4	M	56	None	150 mg BID 2 mg daily	75 mg BID 0.5 mg daily	CR (–100%)	18	None identified
Patient 5	M	49	None	150 mg BID 2 mg daily	150 mg BID 2 mg daily	PR (–45%)	7	None identified

Abbreviation: BID, twice a day.

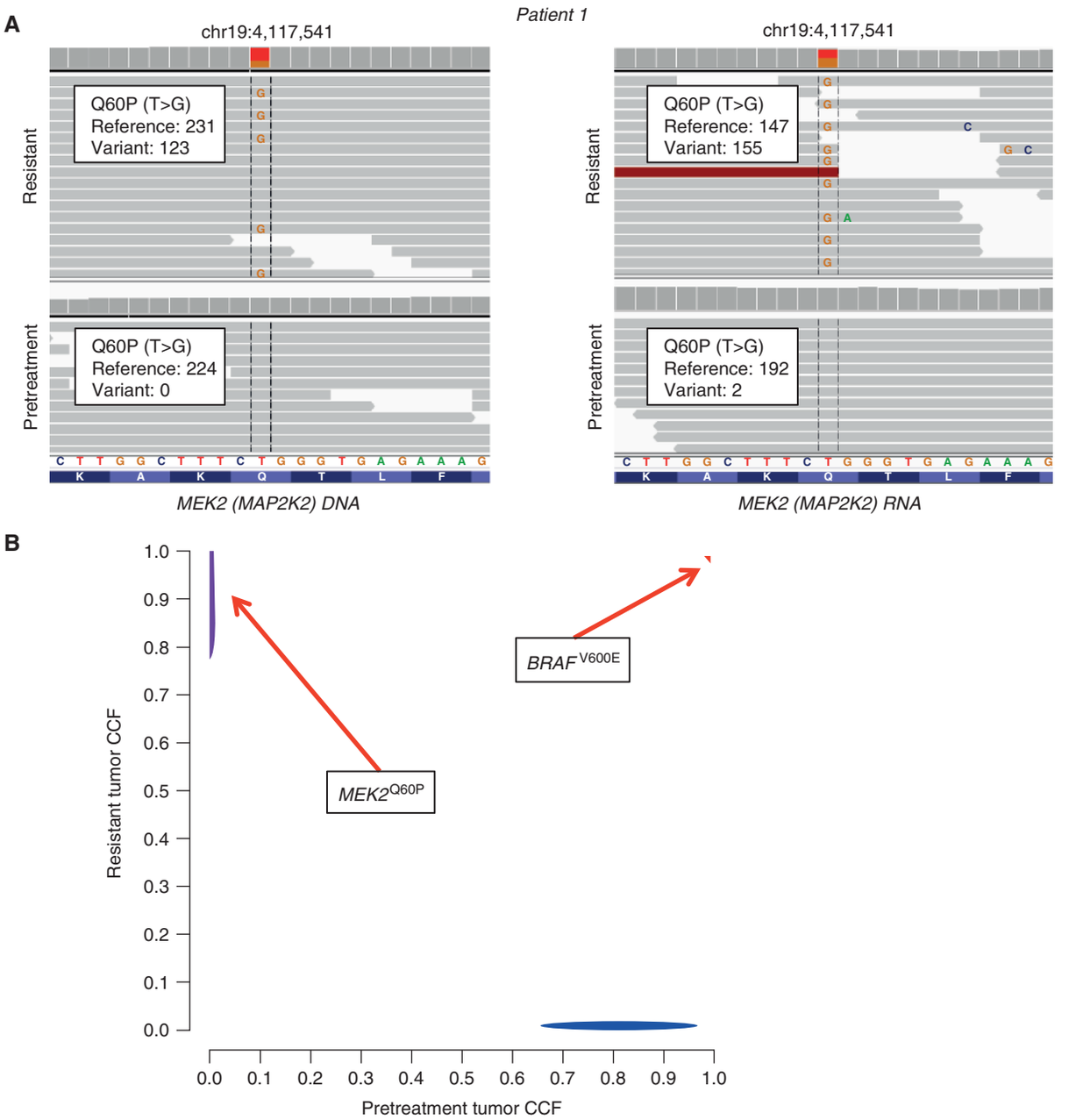

Figure 1. Identification of a *MEK2* mutation in a melanoma sample resistant to dabrafenib/trametinib. **A,** WES (left) and RNA-seq (right) of the tumor tissue from patient 1 both before treatment and after the development of resistance to dabrafenib/trametinib revealed a Q60P mutation in *MEK2* in the resistant tumor that was undetectable in the pretreatment tumor. **B,** the fraction of tumor cells (CCF) harboring each alteration was calculated for the pretreatment and resistant tumor samples. Direct comparison of the CCF for all alterations in the pretreatment and resistant tumor samples demonstrated alterations that occurred in the pretreatment sample only (bottom right, blue), the resistant sample only (top left, purple), or both samples (top right, red). Fifteen missense mutations were identified as occurring in the resistant sample only (top left, purple), including the *MEK2*^{Q60P} mutation (see Supplementary Table S3).

To confirm that *MEK2*^{Q60P} confers resistance to combined RAF/MEK inhibition, the Q60P mutation was introduced into the sequence of wild-type *MEK2* and the mutant cDNA was expressed in a *BRAF*^{V600E} melanoma cell line (A375). Compared with parental controls and cells expressing wild-type *MEK2*, the *MEK2*^{Q60P} mutation conferred profound resistance to the combination of dabrafenib plus trametinib (Fig. 2A), as well as to single-agent dabrafenib (Fig. 2B) and trametinib (Fig. 2C). On the other hand, *MEK2*^{Q60P} did not confer resistance to treatment with an extracellular signal–regulated kinase (ERK) inhibitor (Fig. 2D), which targets the MAPK pathway downstream of MEK1/2. Cells expressing *MEK2*^{Q60P}

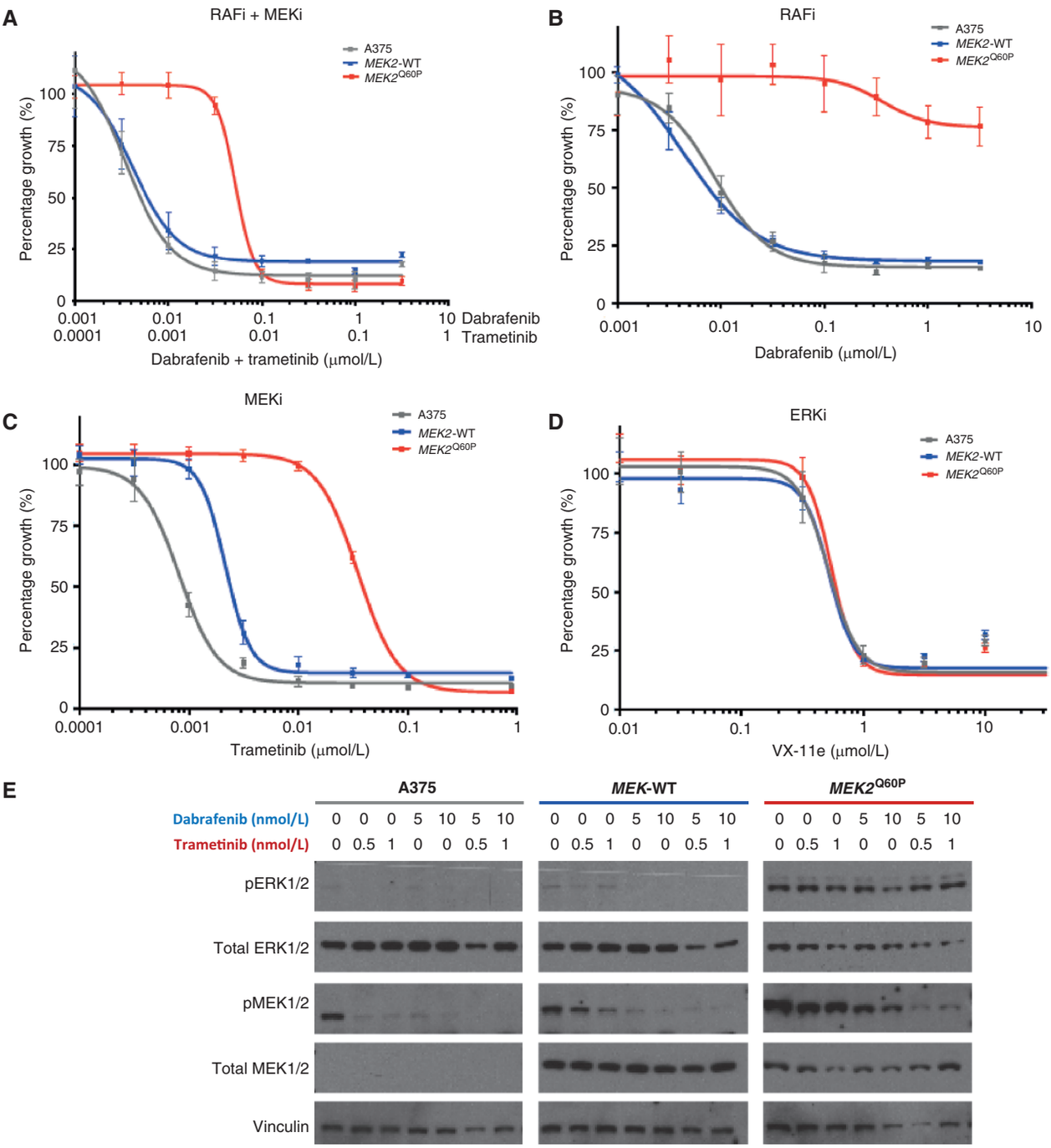

Figure 2. Pharmacologic and biochemical characterization of the *MEK2*^{Q60P} mutation. **A–D,** growth inhibition curves are shown for dabrafenib/trametinib (**A**), as well as monotherapy with dabrafenib (**B**) or trametinib (**C**), and the ERK inhibitor VX-11e (**D**) in A375 (*BRAF*^{V600E}) melanoma cells (gray) and A375 cells expressing wild-type *MEK2* (*MEK2*-WT; blue) or *MEK2*^{Q60P} (red). **E,** the effect of combined dabrafenib/trametinib treatment on ERK1/2 phosphorylation (pERK1/2) in wild-type A375 cells (*BRAF*^{V600E}) and those expressing wild-type *MEK2* (*MEK2*-WT) or *MEK2*^{Q60P} is shown. The levels of pERK1/2, total ERK1/2, pMEK1/2, total MEK1/2, and vinculin are shown after a 16-hour incubation at various drug concentrations as indicated.

exhibited higher levels of phosphorylated ERK1/2 at baseline and when treated with dabrafenib/trametinib than wild-type A375 cells or those expressing wild-type *MEK2* (Fig. 2E), indicative of enhanced MAPK pathway activation despite combined therapeutic blockade of this pathway.

Additional MAPK Pathway Resistance Effectors in Tumors Resistant to Combined Inhibition

Unexpectedly, in 2 patients, WES and RNA-seq revealed additional alterations in the MAPK pathway that have been previously described in patients with acquired resistance to

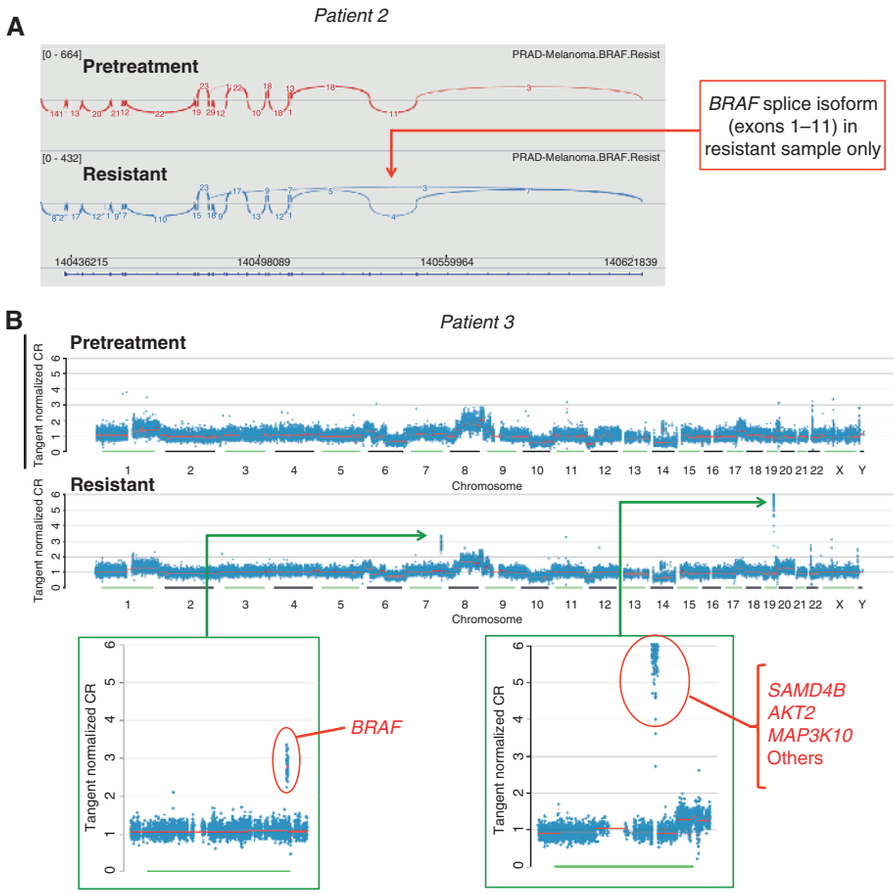

Figure 3. Additional secondary alterations in the MAPK pathway identified in resistant tumors. **A,** RNA-seq of the tumor tissue from patient 2 both before treatment and after the development of resistance to dabrafenib/trametinib revealed a *BRAF* splice isoform lacking exons 2–10 in the resistant tumor that was undetectable in the pretreatment tumor. **B,** copy number analysis of whole-exome data from patient 3 demonstrates two highly amplified regions in the resistant tumor that are not amplified in the pretreatment tumor. One of these regions contains the *BRAF* gene, whereas the second region contains multiple genes, including *SAMD4B*.

vemurafenib monotherapy (Table 1). RNA-seq of the resistant tumor from patient 2 revealed a *BRAF* variant that lacks exons 2–10, which was not detected in the pretreatment tumor (Fig. 3A). Exons 2–10 did not seem to be deleted in the WES data from the resistant sample based on copy number analysis (see Supplementary Methods), suggesting that this represents an alternative *BRAF* splice variant. This splice variant had previously been observed in patients with acquired resistance to single-agent vemurafenib (9). Resistance-associated *BRAF* splice variants lack the RAS-binding domain and allow RAS-independent BRAF dimerization, resulting in downstream ERK activation despite RAF or MEK inhibition (9). No additional MAPK pathway alterations were detected in the resistant tumor from patient 2 by either WES or RNA-seq.

In patient 3, WES revealed a focal *BRAF* chromosomal amplification in the resistant tumor that was absent in the pretreatment tumor (Fig. 3B). *BRAF* amplification has been implicated in acquired resistance to RAF inhibitors (10) or MEK inhibitors (15) as single agents but not in combination.

In an additional patient (patient 4), WES revealed a novel *MEK1* (also known as *MAP2K1*) mutation (*MEK1*^{P162S}) in both the pretreatment and resistant tumor. Certain *MEK1* mutations have been shown to confer acquired resistance to monotherapy with RAF or MEK inhibitors both *in vitro* and in the clinical setting (5, 8, 11). This mutation was detected at high frequency (greater than 50% cancer cell fraction, CCF; see Methods) in both the pretreatment and resistant tumors, despite the patient's CR to therapy (Supplementary Tables S1 and S2). Moreover, *MEK1*^{P162S} did not confer resistance to RAF and/or MEK inhibition *in vitro* (Supplementary Fig. S2). This finding is consistent with prior findings that some tumors with *MEK1* mutations can respond to RAF inhibition (16).

Additional Resistance-Associated Alterations Found by WES and RNA-Seq

Conceivably, drug-resistant tumors may harbor multiple resistance alterations, including new mechanisms discoverable through WES or RNA-seq. To identify additional putative resistance mechanisms in this cohort, we started with the list of point mutations and small insertions and deletions (indel) found in each tumor sample (Supplementary Table S1). This list was then cross-referenced with a set of 198 genes found to confer resistance to combined RAF/MEK inhibition when overexpressed *in vitro* (17) or when silenced in a genome-wide RNA interference (RNAi) screen (ref. 18; Supplementary Table S4), to generate a list of potential resistance alterations in each patient (Supplementary Table S5). These alterations were further filtered to highlight only those alterations that were significantly enriched in the resistant samples compared with their pretreatment counterparts (Supplementary Table S3).

This approach revealed several alterations that may have contributed to acquired resistance in these patients. For example, a mutation in the ETS transcription factor ETS2 (*ETS2*^{P53S}) was identified in the resistant tumor from patient

2 but not in the pretreatment tumor (Supplementary Table S3). ETS2 is a transcription factor target of the MAPK pathway (19, 20) and a validated resistance gene *in vitro*, as overexpression of wild-type ETS2 in *BRAF*-mutant cell lines resulted in resistance to dabrafenib/trametinib (17). This suggests that dysregulated ETS2 activity might engage a gene expression program capable of promoting resistance to upstream MAPK pathway inhibition.

Similarly, in addition to the aforementioned *BRAF* amplification, the resistant tumor from patient 3 contained a second highly amplified region consisting of multiple genes, including *SAMD4B* (Fig. 3B). *SAMD4B*, a poorly characterized gene thought to mediate transcriptional repression (21), was also identified and validated as a resistance gene *in vitro* (17). *SAMD4B* was mutated in the corresponding WES data and showed enriched expression of the mutant allele in the resistant tumor (387 variant reads and 39 reference reads in the resistant tumor, as compared with 36 variant reads and 28 reference reads in the pretreatment tumor). Overexpression of wild-type *SAMD4B* in *BRAF*-mutant cell lines resulted in resistance to combined RAF/MEK inhibition (17). Future functional studies will investigate the mechanism by which mutations in *ETS2*, *SAMD4B*, and other candidate genes can contribute to resistance to RAF/MEK inhibition.

In patients 4 and 5, several alterations were significantly enriched from the pretreatment to the resistant tumors (Supplementary Tables S2, S3, and S5). In patient 4, of 543 coding somatic point mutations and indels found in one or both of the tumors (Supplementary Table S2), 49 alterations were found to be significantly enriched from pretreatment to resistant tumor (Supplementary Table S3). None of these mutations occurred in previously identified resistance genes or known MAPK genes (*NRAS*, *BRAF*, *CRAF*, *MEK1/2*, and *ERK1/2*), nor were they present in the functional screens described above (Supplementary Table S4). Similarly, in patient 5, of 212 coding somatic point mutations and indels (Supplementary Table S2), 20 alterations were found to be significantly enriched from pretreatment to resistant tumor (Supplementary Table S3). None of these were either previously identified resistance genes or present in the functional screens (Supplementary Table S4). Taken together, these results raise the possibility that these tumors may have elaborated as-yet uncharacterized genetic or nongenetic RAF/MEK resistance mechanisms.

DISCUSSION

This case series comprises an initial clinical genomic study of acquired resistance to combined RAF/MEK inhibition in $BRAF^{V600}$-mutant melanoma. Because mechanisms of resistance may occur through both somatic genetic and transcriptional mechanisms, the use of both WES and RNA-seq to identify mutations, copy number alterations, fusions, splice isoforms, and allele-specific expression differences between the pretreatment and resistant tumors potentially offers a more comprehensive view of resistance to combined RAF/MEK inhibition than DNA-based characterization alone.

In post-progression tumors from 3 of 5 patients with acquired resistance to dabrafenib/trametinib, we identified alterations in MAPK genes that were not detected in the pretreatment tumors. Two tumors contained MAPK alterations that had previously been described in patients with acquired resistance to RAF or MEK inhibitor monotherapy. An additional resistant tumor harbored an activating mutation in *MEK2*, which has not previously been implicated in resistance to RAF or MEK inhibition, although the homolog MEK1 has been implicated in resistance to RAF inhibitor and MEK inhibitor monotherapy (5, 8, 11). MEK2 represents a logical resistance mechanism: mutations in this kinase may abrogate the effects of dabrafenib (which acts immediately upstream in the RAF/MEK signaling module) while simultaneously overcoming allosteric MEK inhibition by trametinib. We also recently identified additional *MEK2* mutations in patients with acquired resistance to RAF inhibitor monotherapy (14).

Novel candidate resistance alterations were also identified in some tumors, including cases with known monotherapy-related resistance mechanisms. This suggests that multiple resistance mechanisms may occur in a single tumor sample. Despite this finding, we were not able to identify any obvious candidate mechanisms of resistance in 2 patients, using validated functional screens and well-known MAPK genes as a primary filter. Additional functional follow-up of genomic or transcriptional alterations arising in resistant tumor samples from all 5 of these patients (Supplementary Table S3) may identify additional resistance effectors. Alternatively, additional mechanisms of resistance in these patients may have occurred through other modes not identifiable in the WES and RNA-seq data, such as stromally secreted factors (22) or posttranslational effects.

This is the first study, to our knowledge, of clinically acquired resistance to combined targeted therapy in cancer. One of the expected advantages of combining targeted therapies in genetically defined tumor contexts is the theoretical ability to overcome common mechanisms of resistance to monotherapies. In melanoma, targeting the MAPK pathway with dual RAF and MEK inhibition was expected to overcome common MAPK-based resistance mechanisms seen with vemurafenib, dabrafenib, or trametinib alone. Although combined RAF/MEK inhibition may indeed prevent resistance due to activating mutations in *NRAS*, which we did not identify in this study, it was somewhat surprising to find alterations in BRAF, which might have been expected to be overcome by adequate MEK inhibition, emerge in the resistant tumors from 2 patients.

These results indicate that at least some MAPK pathway alterations arising in the setting of monotherapy (*MEK1/2* mutations, *BRAF* amplification, *BRAF* splice isoforms) are also likely to cause cross-resistance to combination therapy. Indeed, several *MEK1* and *MEK2* mutations (e.g., $MEK1^{C121S}$, $MEK1^{G128V}$, $MEK2^{C125S}$, and $MEK2^{L46F}$; refs. 8, 14) confer resistance to combination dabrafenib/trametinib *in vitro*, even though the patients in whom those mutations were identified had never been exposed to that combination (Supplementary Fig. S3). This result may help to provide a mechanistic basis for the much higher proportion of patients with intrinsic resistance to dabrafenib/trametinib when this combination is used following single-agent RAF or MEK inhibitors (23), further supporting the use of combined RAF/MEK inhibition as first-line therapy.

As with resistance to single-agent RAF inhibition (6–11), the prevalence of MAPK pathway alterations in these resistant tumors indicates that *BRAF*-mutant melanomas remain

dependent on MEK/ERK signaling despite combined pathway inhibition. Conceivably, more potent MEK inhibition might circumvent some of these resistance mechanisms—although toxicity concerns have constrained the dosing of MEK inhibitor monotherapy. In the future, small-molecule ERK inhibitors, now in clinical trials, may provide an additional avenue for overcoming RAF- or MEK-centered resistance mechanisms.

Finally, the identification of somatic mutations in genes such as *ETS2* and *SAMD4B* is noteworthy for two reasons. First, these observations highlight the potential value of integrating systematic functional data pertaining to drug resistance derived from preclinical studies with the results of deep genomic characterization of clinical tumor specimens obtained before treatment with targeted therapies and following relapse. This type of integrative approach is likely to provide a valuable means for cross-filtering and prioritization of candidate mechanisms identified from preclinical or clinical analyses individually.

Second, these findings raise the possibility that mechanisms outside the canonical MAPK pathway may also emerge as RAF/MEK resistance effectors in the future. Of course, the specific functional effects of these mutations will need to be examined mechanistically to clarify their importance in driving resistance phenotypes. Nonetheless, the observation supports the notion that clinical testing of higher-order therapeutic combinations directed against other signaling pathways as well as immunotherapy should be prioritized in addition to MAPK-directed therapy. The use of serial biopsies and genomic/molecular profiling at the time of resistance may ultimately improve the care of patients with resistant *BRAF*-mutant melanoma through tailored targeted combinations to overcome specific resistance mechanisms.

METHODS

Patients and Tumor Samples

We obtained pretreatment and drug-resistant tumor specimens along with normal blood samples from 5 patients with acquired resistance to dabrafenib/trametinib. All patients provided written informed consent to genomic profiling of tumor and normal DNA/RNA, as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-181).

WES and RNA-Seq Analysis

WES was performed on pretreatment tumors, resistant tumors, and normal samples from all 5 patients, as detailed in the Supplementary Data. The mean depth of coverage for the tumor samples was 255X (range, 125X–338X; Supplementary Table S6). Sequencing data were analyzed using tools to identify somatic point mutations, small indels, and copy number alterations (see Supplementary Data).

RNA-seq was performed on pretreatment and resistant tumors from 4 patients, as detailed in the Supplementary Data and Supplementary Table S6. RNA-seq of the pretreatment samples from patients 4 and 5 could not be completed because of poor-quality RNA. For patient 4, RNA was obtained from a second biopsy taken 1 week after the start of therapy, and this was used as a substitute for the pretreatment sample in the RNA-Seq analysis for this patient. Transcriptome data were analyzed for rearrangements/fusions that were enriched in the resistant tumor as compared with the pretreatment tumor. In addition, transcriptome data were analyzed specifically for alternatively spliced isoforms from the MAPK pathway genes *BRAF*, *NRAS*, *MEK1*, and *MEK2*.

To prioritize candidate resistance alterations, we highlighted those somatic point mutations or indels that were novel or significantly enriched in the resistant samples as compared with each matched pretreatment sample (24). This was done by estimating the fraction of tumor cells (CCF) harboring a given alteration in each pair of samples using the ABSOLUTE algorithm (see Supplementary Data). Transcriptome data were queried to determine whether these specific DNA alterations were expressed in the pretreatment and resistant tumors. ABSOLUTE for patient 3 could not be completed for technical reasons; for this patient, the pretreatment and resistant WES and RNA-Seq data were manually compared.

Detailed analyses of all sequencing results are available in Supplementary Tables S1–S6 and Supplementary Fig. S1.

Experimental Analysis

Expression plasmids containing *MEK1* and *MEK2* cDNA were generated and site-directed mutagenesis was performed as detailed in the Supplementary Data. Viral infections, cell growth inhibition analysis, and immunoblot studies were performed using standard protocols (see Supplementary Data). Cell lines were obtained from the American Type Culture Collection, which verifies identity by short-tandem repeat profiling, and were passaged less than 6 months following receipt. Physical and biologic containment procedures for recombinant DNA followed institutional protocols in accordance with the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

Disclosure of Potential Conflicts of Interest

N. Wagle has ownership interest (including patents) in Foundation Medicine and is a consultant/advisory board member of the same. L.A. Garraway has received a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member of Novartis, Foundation Medicine, Boehringer-Ingelheim, and Millennium. No potential conflicts of interest were disclosed by the other authors.

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