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 (MEK2Q60P). MEK2Q60P 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): 1–8. ©2013 AACR.
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 (trametinib) 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 trametinib, 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 MEK2Q60P 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, MEK2Q60P, 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). MEK2Q60P is homologous to MEK1Q60P, 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

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Prior systemic therapy for metastatic melanoma</th>
<th>Dabrafenib dosing</th>
<th>Trametinib dosing</th>
<th>Best response</th>
<th>Duration of response, mo</th>
<th>MAPK pathway candidate resistance mechanisms</th>
</tr>
</thead>
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<tr>
<td>Patient 1</td>
<td>M</td>
<td>72</td>
<td>None</td>
<td>150 mg BID 2 mg daily</td>
<td>150 mg BID 2 mg daily</td>
<td>PR (–64%)</td>
<td>3</td>
</tr>
<tr>
<td>Patient 2</td>
<td>M</td>
<td>48</td>
<td>None</td>
<td>150 mg BID 2 mg daily</td>
<td>100 mg BID 2 mg daily</td>
<td>PR (–42%)</td>
<td>3</td>
</tr>
<tr>
<td>Patient 3</td>
<td>M</td>
<td>42</td>
<td>None</td>
<td>150 mg BID 1 mg daily</td>
<td>150 mg BID 1 mg daily</td>
<td>SD (–19.5%)</td>
<td>11</td>
</tr>
<tr>
<td>Patient 4</td>
<td>M</td>
<td>56</td>
<td>None</td>
<td>150 mg BID 2 mg daily</td>
<td>75 mg BID 0.5 mg daily</td>
<td>CR (–100%)</td>
<td>18</td>
</tr>
<tr>
<td>Patient 5</td>
<td>M</td>
<td>49</td>
<td>None</td>
<td>150 mg BID 2 mg daily</td>
<td>150 mg BID 2 mg daily</td>
<td>PR (–45%)</td>
<td>7</td>
</tr>
</tbody>
</table>

Abbreviation: BID, twice a day.
MAPK Alterations in Melanoma Resistant to RAF/MEK Inhibition

**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<sup>Q60P</sup> 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<sup>V600E</sup> melanoma cell line (A375). Compared with parental controls and cells expressing wild-type MEK2, the MEK2<sup>Q60P</sup> 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<sup>Q60P</sup> 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<sup>Q60P</sup>...
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
MAPK Alterations in Melanoma Resistant to RAF/MEK Inhibition

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 (MEK1P162S) 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, MEK1P162S 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 (ETS2P535S) was identified in the resistant tumor from patient...
was also identified and validated as a resistance gene thought to mediate transcriptional repression (21), as it was mutated in the corresponding WES data (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 evolved 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 BRAFV600E-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).

In patients 4 and 5, several 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<sup>G121S</sup>, MEK1<sup>G120V</sup>, MEK2<sup>C125S</sup>, and MEK2<sup>A68S</sup>; 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). Transcriptome data were analyzed using tools to identify somatic point mutations, small indels, and copy number alterations (see Supplementary Data). 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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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Wagle, E.M. Van Allen, D.J. Treacy, D.T. Frederick, Z.A. Cooper, E.M. Goetz, R.J. Sullivan, D.C. Friedrich, K. Anderka, C.M. Johannessen, D.P. Lawrence, S.B. Gabriel, K.T. Flaherty, J.A. Wargo, L.A. Garraway


Writing, review, and/or revision of the manuscript: N. Wagle, E.M. Van Allen, D.T. Frederick, Z.A. Cooper, R.J. Sullivan, D. Perrin, D.P. Lawrence, K.T. Flaherty, J.A. Wargo, L.A. Garraway

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Wagle, E.M. Van Allen, A. Taylor-Weiner, D.N. Farlow


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