ABSTRACT

BRAF mutations occur in approximately 10% of colorectal cancers. Although RAF inhibitor monotherapy is highly effective in BRAF-mutant melanoma, response rates in BRAF-mutant colorectal cancer are poor. Recent clinical trials of combined RAF/EGFR or RAF/MEK inhibition have produced improved efficacy, but patients ultimately develop resistance. To identify molecular alterations driving clinical acquired resistance, we performed whole-exome sequencing on paired pretreatment and postprogression tumor biopsies from patients with BRAF-mutant colorectal cancer treated with RAF inhibitor combinations. We identified alterations in MAPK pathway genes in resistant tumors not present in matched pretreatment tumors, including KRAS amplification, BRAF amplification, and a MEK1 mutation. These alterations conferred resistance to RAF/EGFR or RAF/MEK combinations through sustained MAPK pathway activity, but an ERK inhibitor could suppress MAPK activity and overcome resistance. Identification of MAPK pathway reactivating alterations upon clinical acquired resistance underscores the MAPK pathway as a critical target in BRAF-mutant colorectal cancer and suggests therapeutic options to overcome resistance.

SIGNIFICANCE: RAF inhibitor combinations represent promising approaches in clinical development for BRAF-mutant colorectal cancer. Initial characterization of clinical acquired resistance mechanisms to these regimens identified several MAPK pathway alterations driving resistance by reactivating MAPK signaling, highlighting the critical dependence of BRAF-mutant colorectal cancers on MAPK signaling and offering potential strategies to overcome resistance.

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See related commentary by Meador and Pao, p. 348.
activate ERK kinases, which phosphorylate a multitude of key cellular substrates involved in cell proliferation and survival.

RAF inhibitors, such as vemurafenib and dabrafenib, have produced response rates of approximately 50% to 80% in the roughly half of melanomas that also harbor BRAFV600E mutations, revolutionizing the treatment of these cancers and leading to the FDA approval of vemurafenib and dabrafenib for this disease (4, 5). However, in colorectal cancers harboring the identical BRAFV600E mutation, RAF inhibitor mono-therapy has proven disappointingly ineffective, with response rates of only approximately 5% (6). This striking disparity in sensitivity between these two tumor types represents a critical challenge to the development of effective therapies for BRAF-mutant colorectal cancer. Previous work by our group and others suggested that RAF inhibitor insensitivity in BRAF-mutant colorectal cancer is driven by feedback reactivation of MAPK signaling following RAF inhibitor treatment (7–9). In many, but not all, BRAF-mutant colorectal cancers, the feedback reactivation of MAPK signaling is driven by EGFR-mediated activation of RAS and CRAF (8, 10). Preclinical studies have suggested that RAF inhibitor combination strategies, including cotargeting RAF and EGFR or RAF and MEK, can suppress feedback reactivation of MAPK signaling, leading to more robust and sustained inhibition of the pathway and to improved efficacy in BRAF-mutant colorectal cancer.

Based on these data, RAF inhibitor combinations have been evaluated in clinical trials for patients with BRAF-mutant colorectal cancer in recent years and are showing signs of improved efficacy compared with RAF inhibition alone. A clinical trial of combined RAF/MEK inhibition with dabrafenib and the MEK inhibitor trametinib in 43 patients with BRAFV600E colorectal cancer produced a response rate of 12%, including 1 patient with a durable complete response that remains ongoing over 3 years. In addition, 51% of patients achieved disease stabilization on study (11). More recently, clinical trials of combined RAF/EGFR inhibition have been initiated, including the combination of vemurafenib and the anti-EGFR antibody cetuximab, the combination of the RAF inhibitor encorafenib (LX-818) and cetuximab, and the combination of dabrafenib and the anti-EGFR antibody panitumumab (12–14). Some of these trials have produced preliminary response rates of as high as 29%. A trial of combined RAF/EGFR/MEK inhibition with the triple combination of dabrafenib, panitumumab, and trametinib is also under way and has shown an initial response rate of 40% (12). However, despite the promising signs of efficacy demonstrated by early clinical trials of RAF inhibitor combinations in patients with BRAF-mutant colorectal cancer, those patients deriving initial benefit from therapy ultimately develop resistance to treatment and disease progression. An understanding of the mechanisms of clinical acquired resistance that arise to RAF inhibitor combinations in patients with BRAF-mutant colorectal cancer may lead to valuable opportunities to overcome resistance and prolong clinical response.

Here, we find that MAPK pathway alterations leading to reactivation of MAPK signaling are important drivers of acquired resistance in BRAF-mutant colorectal cancer. In vitro modeling of acquired resistance to RAF/EGFR or RAF/MEK inhibitor combinations in BRAF-mutant colorectal cancer cell lines revealed activating KRAS exon 2 mutations as drivers of acquired resistance to either therapy through sustained MAPK pathway activation. Whole-exome sequencing (WES) of paired pretreatment and postprogression biopsies from patients with initial clinical response or prolonged stable disease on RAF/EGFR or RAF/MEK combination therapy identified MAPK pathway alterations unique to the resistant tumor, including KRAS amplification, BRAF amplification, and MEK1 mutation. Each of these alterations led to resistance to RAF/EGFR or RAF/MEK combinations and to sustained MAPK pathway activation in vitro, but retained sensitivity to an ERK inhibitor. These findings highlight the critical importance of the MAPK pathway as a therapeutic target in BRAF-mutant colorectal cancer and point to possible therapeutic strategies to overcome clinical resistance.

RESULTS

In Vitro Modeling Identifies RAS Activation as a Potential Resistance Mechanism

To begin to understand the spectrum of molecular alterations that can drive acquired resistance to RAF inhibitor combinations, we generated resistant variants of the sensitive BRAF-mutant colorectal cancer cell line VACO432 by culturing cells in the presence of a RAF/EGFR inhibitor combination using vemurafenib and cetuximab (VACO-RE cells) or a RAF/MEK combination using vemurafenib and selumetinib (VACO-RM cells). Interestingly, resistant cells derived using one RAF inhibitor combination were cross-resistant to the other RAF inhibitor combination (Fig. 1A). Sequence analysis of VACO-RE and VACO-RM cells revealed that each resistant variant retained the original BRAFV600E mutation, but that each acquired a distinct exon 2 mutation in KRAS (G12D and G13D, respectively) that was not present in the parental cell line, with no change in KRAS copy number (Fig. 1B and Supplementary Fig. S1). The identification of KRAS mutations as a potential mechanism of acquired resistance is consistent with previous findings that NRAS mutations are a common cause of clinical acquired resistance in BRAF-mutant melanoma (15, 16). Exogenous expression of KRASG12D or KRASG13D in VACO432 cells recapitulated resistance to RAF/EGFR and RAF/MEK inhibitor combinations, including combinations currently in clinical trials (Fig. 1C and Supplementary Fig. S2A). Expression of mutant KRAS led to increased basal levels of phosphorylated CRAF (pCRAF), pMEK, and pERK (Fig. 1D). Importantly, mutant KRAS expression also abrogated the ability of RAF/EGFR or RAF/MEK combinations to inhibit MAPK signaling, as evidenced by sustained phosphorylation of ERK and of the ERK target RSK (pRSK). These findings suggest that activation of RAS can maintain MAPK pathway signaling and drive resistance to either combined RAF/EGFR or RAF/MEK inhibition.

Interestingly, an ERK inhibitor retained the ability to suppress MAPK despite expression of KRASG12D or KRASG13D, as measured by its ability to inhibit pRSK levels (because certain ERK inhibitors like VX-11e lead to a feedback induction of pERK despite inhibition of ERK kinase activity; refs. 17, 18), and was able to overcome resistance (Fig. 1E and F and Supplementary Fig. S2B–S2D). These results emphasize the importance of sustained MAPK signaling in driving resistance to RAF inhibitor combinations in BRAF-mutant colorectal cancer cells and suggest that therapies that restore
MAPK pathway suppression may be promising strategies to overcome resistance.

**Clinical Acquired Resistance to Combined RAF/EGFR Inhibition**

To identify acquired resistance mechanisms to RAF inhibitor combinations that occur clinically, we evaluated paired tumor biopsies taken before treatment and after eventual disease progression from patients with BRAF-mutant colorectal cancer who achieved an initial response or prolonged stable disease on a RAF/EGFR or RAF/MEK inhibitor combination (Supplementary Table S1). Pretreatment and postprogression tumor biopsies were evaluated by WES to identify novel molecular alterations arising in the drug-resistant postprogression tumor that were absent in the pretreatment tumor. Paired biopsies from 2 patients with clinical acquired resistance to combined RAF/EGFR inhibition were evaluated.

The first patient with BRAF-mutant colorectal cancer was initially treated with the RAF/MEK combination of dabrafenib and trametinib and achieved a prolonged minor response lasting over 7 months, as evidenced by the decrease in tumor burden as measured by RECIST and the decrease in the patient’s serum carcinoembryonic antigen (CEA) tumor marker (Fig. 2A). Ultimately, the patient progressed on RAF/MEK combination...
Clinical Acquired Resistance in BRAF-Mutant Colorectal Cancer

RESEARCH BRIEF

Figure 2. KRAS amplification can drive clinical acquired resistance to combined RAF/EGFR or RAF/MEK inhibition. A, clinical time course of therapy for patient #1 with BRAF-mutant colorectal cancer showing dates of therapy and timing of biopsies. Biopsies #1 (post–RAF/MEK) and #2 (post–RAF/EGFR) were taken from distinct metastatic lesions. Serum CEA tumor marker levels and cumulative tumor diameter as measured by RECIST are shown throughout the treatment course. B, RECIST measurements of individual target lesions during therapy with encorafenib plus cetuximab from April 30 to September 23, 2013. Target lesion #3 was biopsied upon progression. C, CT images of target lesion #3 (indicated by yellow arrow) throughout therapy with dabrafenib plus trametinib and encorafenib plus cetuximab. D, DNA copy-number traces for chromosome 12 showing focal amplification of KRAS in the post–RAF/EGFR biopsy. KRAS transcript abundance as determined by RNA-seq is also shown for each sample. CR, copy ratio; RPKM, reads per kilobase of transcript per million mapped reads. E, FISH was performed on biopsy specimens using probes for KRAS (red) and chromosome 12 (Chr12; green). F, VACO432 cells overexpressing yellow fluorescent protein (YFP) control or wild-type KRAS (KRAS WT ) were treated for 72 hours with the indicated concentrations of drug, and relative cell titer was determined. G, VACO432 cells overexpressing YFP or KRAS WT were lysed, and Western blotting was performed with the indicated antibodies. H, Western blot analysis of VACO432 cells overexpressing YFP or KRAS WT treated with the indicated concentrations of drug for 24 hours was performed.
therapy, and at that time the patient underwent complete excision of a rapidly growing subcutaneous lesion near the umbilicus (post–RAF/MEK biopsy). The patient was then treated with the RAF/EGFR inhibitor combination of encorafenib and cetuximab and experienced a dramatic drop in the CEA tumor marker levels after initiating therapy, suggesting an overall decrease in tumor burden (Fig. 2A). The patient also experienced a decrease in two of three target lesions by RECIST (Fig. 2B). However, one target lesion progressed steadily through therapy, eventually causing the patient to meet RECIST criteria for progressive disease. Interestingly, this lesion was present while the patient was receiving RAF/MEK combination therapy, and although the lesion remained stable during the first few months of RAF/MEK therapy, the lesion began to progress during the later months of RAF/MEK treatment (Fig. 2C). This suggests that perhaps the molecular alteration driving resistance in this lesion actually may have arisen as a mechanism of acquired resistance during RAF/MEK therapy and then caused this specific lesion to be immediately refractory to subsequent RAF/EGFR therapy, even though the remainder of the patient’s disease appeared to respond to combined RAF/EGFR inhibition.

This progressing lesion (post–RAF/EGFR) was biopsied and was evaluated by WES and RNA sequencing (RNA-seq) compared with both the patient’s primary tumor and the separate metastatic lesion excised after progression on combined RAF/MEK therapy (post–RAF/MEK). The post–RAF/MEK biopsy retained the original BRAF\textsuperscript{V600E} mutation, but harbored no new mutations compared with the primary tumor, and a definitive mechanism of resistance was not identified (Supplementary Fig. S3). The post–RAF/EGFR progression biopsy retained the original BRAF\textsuperscript{V600E} mutation, but no new candidate resistance mutations arising specifically in the post–RAF/EGFR biopsy were identified (Supplementary Table S2). However, copy-number analysis revealed focal amplification of KRAS on chromosome 12 in this resistant lesion that was not present in either of the two prior biopsy specimens (Fig. 2D). Amplification of wild-type KRAS has previously been implicated as a mechanism of resistance to targeted therapies, including anti-EGFR antibodies like cetuximab (19). RNA-seq confirmed approximately 6- to 8-fold overexpression of KRAS transcript in the post–RAF/EGFR biopsy relative to each of the prior biopsies. FISH confirmed approximately 25-fold amplification of KRAS in the post–RAF/EGFR biopsy (Fig. 2E), suggesting KRAS amplification as the likely driver of acquired resistance in this lesion.

Overexpression of wild-type KRAS conferred resistance to multiple RAF/EGFR inhibitor combinations (Fig. 2F and Supplementary Fig. S4A). Notably, KRAS overexpression also conferred resistance to RAF/MEK inhibitor combinations (Supplementary Fig. S4B), supporting the possibility that this alteration may have initially arisen as an acquired resistance mechanism to the patient’s original RAF/MEK therapy and then promoted upfront resistance to subsequent RAF/EGFR therapy. Similar to our observations with mutant KRAS expression, overexpression of wild-type KRAS led to increased basal levels of pCRAF, pMEK, and pERK, and abrogated the ability of combined RAF/EGFR or RAF/MEK inhibition to inhibit the MAPK pathway (Fig. 2G and H and Supplementary Fig. S4C).

Importantly, an ERK inhibitor again was able to suppress the MAPK pathway in cells overexpressing wild-type KRAS and was able to overcome resistance (Fig. 2F and H). Collectively, our in vitro and clinical findings suggest that activation of RAS, either by mutation of KRAS or amplification of wild-type KRAS, can promote resistance to either RAF/EGFR or RAF/MEK inhibitor combinations by promoting sustained MAPK pathway signaling. However, in either case, an ERK inhibitor retains its ability to block MAPK signaling and can suppress resistance conferred by either KRAS alteration.

A second patient with BRAF-mutant colorectal cancer achieved a minor response to combined RAF/EGFR inhibition with dabrafenib and panitumumab, lasting almost 5 months before eventual disease progression (Fig. 3A). A progressing retroperitoneal lymph node tumor was biopsied upon disease progression and evaluated by WES in comparison with a biopsy of the same lesion obtained immediately before the start of therapy. The patient was subsequently treated with the triple RAF/EGFR/MEK inhibitor combination of dabrafenib, panitumumab, and trametinib, but progressed rapidly through therapy.

WES revealed that the postprogression specimen retained the original BRAF\textsuperscript{V600E} mutation, but did not identify any new mutations arising in the postprogression biopsy likely to explain acquired resistance (Supplementary Table S2). However, copy-number analysis identified a focal BRAF amplification on chromosome 7, and BRAF amplification was confirmed by FISH (Fig. 3B and C). The high allele frequency of the BRAF\textsuperscript{V600E} mutation in the postprogression biopsy suggests predominant amplification of the mutant Braf allele (Fig. 3B). Previously, our group identified amplification of mutant Braf as a mechanism of resistance to RAF or MEK inhibition in BRAF-mutant colorectal cancer, and, subsequently, BRAF amplification has been implicated as an important acquired resistance mechanism to RAF inhibitor monotherapy or combined RAF/MEK inhibition in BRAF-mutant melanoma (7, 20, 21). Overexpression of BRAF\textsuperscript{V600E} led to increased basal levels of pMEK and pERK in BRAF-mutant colorectal cancer cells and conferred resistance to combined RAF/EGFR inhibition (Fig. 3D and E). Notably, BRAF\textsuperscript{V600E} overexpression also led to cross-resistance to combined RAF/MEK and combined RAF/EGFR/MEK inhibition, perhaps explaining why the patient did not respond to the triple combination after initial disease progression on RAF/EGFR therapy (Fig. 3E). BRAF\textsuperscript{V600E} overexpression abrogated the ability of combined RAF/EGFR or RAF/MEK inhibition to suppress MAPK signaling (Fig. 3F). However, an ERK inhibitor retained the ability to suppress the MAPK pathway and to overcome resistance caused by BRAF\textsuperscript{V600E} overexpression (Fig. 3E and F).

**Clinical Acquired Resistance to Combined RAF/MEK Inhibition**

A third patient with BRAF-mutant colorectal cancer was treated with combined RAF/MEK inhibition with dabrafenib and trametinib. The patient achieved a reduction in tumor burden that met the criteria for a partial response by RECIST, but unfortunately developed disease progression after almost 6 months of therapy (Fig. 4A). The patient was then treated with the combination of a PI3K/mTOR inhibitor and a MEK inhibitor, but progressed rapidly through...
Figure 3. BRAF amplification causes clinical acquired resistance to combined RAF/EGFR inhibition. A, clinical time course of therapy for patient #2 with BRAF-mutant colorectal cancer showing dates of therapy and timing of postprogression biopsy. CEA tumor marker levels and cumulative tumor diameter as measured by RECIST are shown throughout the treatment course. B, DNA copy-number traces are shown for the postprogression biopsy compared with a pretreatment biopsy taken from the same lesion immediately before the start of panitumumab + dabrafenib therapy. Focal amplification of BRAF on chromosome 7 is shown in the postprogression biopsy. BRAF<sup>V600E</sup> mutant allele frequencies are shown for each sample. CR, copy ratio. C, FISH was performed on the pretreatment and postprogression biopsies using probes for BRAF (red) and chromosome 7 (Chr7; green). D, VACO432 cells overexpressing BRAF<sup>V600E</sup> compared with empty vector were lysed, and Western blotting was performed with the indicated antibodies. E, cells from D were treated for 72 hours with the indicated concentrations of dabrafenib (DAB), panitumumab (PAN), trametinib (TRA), or VX-11e (VX) for 72 hours, and relative cell titer was determined. F, cells from D were treated for 24 hours with the indicated concentrations of drugs, and Western blotting was performed with the indicated antibodies.
therapy. WES revealed that a tumor biopsy taken after progression on BRAF/MEK therapy retained the original BRAF V600E mutation, but also identified the presence of two candidate resistance mutations that were not present in the pretreatment biopsy (Fig. 4B and Supplementary Table S2). One mutation was a Q489L missense mutation in another RAF kinase, ARAF. ARAF mutations are rare in human cancer, but Q489 in ARAF corresponds to Q636 in BRAF, which is mutated in a small percentage of lung and colorectal cancers (22). The second mutation was a F53L missense mutation in MEK1 (MAP2K1) that occurs in helix A, a region of MEK1 and MEK2 previously found to be mutated in BRAF-mutant melanomas that have acquired resistance to RAF inhibitors or RAF/MEK inhibitor combinations (16, 21). The lower allele frequencies of the ARAF and MEK1 mutations suggested the possibility that these mutations were subclonal, perhaps representing two populations of cancer cells that developed resistance independently through distinct mechanisms, with each population harboring one mutation. Although it is not possible to determine whether the ARAF and MEK1 mutations coexist in the same tumor cells from the WES alone, we were able to utilize a...

Figure 4. MEK1 F53L mutation drives clinical acquired resistance to combined RAF/MEK inhibition. A, clinical time course of therapy for patient #3 with BRAF-mutant colorectal cancer showing dates of therapy and timing of postprogression biopsy. Cumulative tumor diameter as measured by RECIST is shown throughout the treatment course. B, list of key mutations identified in the postprogression biopsy with associated allele frequencies. C, Sanger sequencing of a patient-derived cell line generated from the postprogression biopsy for BRAF V600E, ARAF Q489L, and MEK1 F53L. All mutations were found to be present in 30 of 30 single-cell clones. D, cells expressing the indicated constructs were treated with dabrafenib (DAB) plus trametinib (TRA) or VX-11e (VX) as shown for 72 hours, and relative cell titer was determined. E, cells expressing wild-type MEK1 (MEK1 WT), MEK1 F53L, or empty vector control were treated with the indicated concentrations of drugs for 24 hours, and Western blotting was performed with the indicated antibodies.
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patient-derived cell line generated from the postprogression biopsy. Sanger sequencing of the pooled cell line successfully identified the presence of the original BRAF\(_{V600E}\) mutation as well as the presence of both candidate resistance mutations (Fig. 4C). We generated 30 single-cell clones to determine if individual tumor cells harbored different combinations of these three mutations. Surprisingly, we found that all 30 single-cell clones harbored all three mutations, suggesting that the clone or clones that gave rise to this cell line harbored both candidate resistance mutations as well as the original BRAF\(_{V600E}\) mutation. Although this result does not preclude the possibility that a subpopulation of tumor cells may have developed one candidate resistance mutation first and that the second mutation may have arisen within a subclone of that population, it does make it highly unlikely that these two mutations existed independently in distinct tumor cells within the resistant lesion.

Consistent with these findings, we found that exogenous expression of ARAF\(_{Q489L}\) did not markedly confer resistance to dabrafenib and trametinib relative to wild-type ARAF, whereas the previously identified activating S214C mutation in ARAF (23) effectively promoted resistance (Fig. 4D). Furthermore, ARAF\(_{S214C}\), but not ARAF\(_{Q489L}\), led to MAPK pathway activation in 293T cells, relative to wild-type ARAF (Supplementary Fig. 5A and 5B). Although it is still possible that ARAF\(_{Q489L}\) may have a subtle contribution to drug sensitivity, this mutation does not appear to be a primary driver of acquired RAF/MEK resistance in this setting.

Conversely, exogenous expression of MEK1\(_{F53L}\) led to robust resistance to dabrafenib and trametinib and to increased basal activation and phosphorylation of ERK, relative to wild-type MEK1 (Fig. 4D and E). MEK1\(_{F53L}\) expression also abrogated the ability of dabrafenib and trametinib to suppress MAPK signaling. Thus, MEK1\(_{F53L}\) is the likely driver of clinical acquired resistance in this patient’s tumor. Importantly, an ERK inhibitor retained the ability to suppress MAPK signaling and could mitigate resistance caused by MEK1\(_{F53L}\) expression. Similarly, an ERK inhibitor, alone or in combination with dabrafenib, was able to mitigate resistance in the tumor cell line derived from this patient (Supplementary Fig. S5C). Because multiple mechanisms of acquired resistance to targeted therapy can arise within an individual patient (16), this case serves as an important example that careful validation of candidate resistance mechanisms will be a critical adjunct to properly interpret sequencing data as it pertains to the potential heterogeneity of acquired resistance.

**DISCUSSION**

In this study, we identified multiple molecular alterations within the MAPK pathway that lead to reactivation of MAPK signaling and acquired resistance to combined RAF/EGFR and RAF/MEK inhibition in BRAF-mutant colorectal cancer, both in vitro and in patient biopsies obtained upon development of clinical acquired resistance. The fact that all resistance mechanisms we identified promote reactivation of the MAPK pathway highlights the critical dependence of BRAF-mutant colorectal cancer on sustained MAPK signaling to maintain cancer cell proliferation and survival and underscores the MAPK pathway as a critical therapeutic target in this disease. Indeed, initial pharmacodynamic analysis of paired pretreatment and on-treatment biopsies from patients with BRAF-mutant colorectal cancer on clinical trials of RAF/EGFR or RAF/MEK inhibitor combinations has suggested that a major factor limiting response is that these inhibitor combinations still produce a lesser degree of MAPK pathway inhibition in patients with BRAF-mutant colorectal cancer than single-agent RAF inhibitors in patients with BRAF-mutant melanoma (11, 12). Conversely, the triple combination of RAF/EGFR/MEK inhibition produces a degree of MAPK inhibition closer to that observed with single-agent RAF inhibitor in BRAF-mutant melanoma, underscoring the importance of robust and sustained MAPK inhibition in BRAF-mutant cancers (12). These data are consistent with previous reports that the majority of acquired resistance mechanisms to RAF or RAF/MEK inhibition in BRAF-mutant melanoma—including NRAS alterations, BRAF amplification, MEK1 or MEK2 mutations, and RAF splice isoforms—also lead to reactivation of MAPK signaling, as opposed to activation of alternative parallel signaling pathways (15, 16, 21). As some of these same resistance mechanisms were identified in our initial analysis of acquired resistance in BRAF-mutant colorectal cancer, including RAS alterations (though in KRAS, not NRAS), BRAF amplification, and MEK1 mutation, it is possible that other known resistance mechanisms in BRAF-mutant melanoma leading to MAPK reactivation may be found in other patients with BRAF-mutant colorectal cancer. Indeed, the continued MAPK pathway dependence of BRAF-mutant colorectal cancer cells harboring molecular alterations promoting acquired resistance is supported by the fact that they remain sensitive to ERK inhibitors, which retain the ability to suppress MAPK signaling despite the presence of these alterations. Although it is certainly possible that MAPK-independent mechanisms may lead to clinical acquired resistance in some cases of BRAF-mutant colorectal cancer, collectively these findings emphasize that effective targeting of the MAPK pathway is paramount in the therapeutic approach to this disease.

Although our findings support MAPK pathway reactivation as a key event in the development of acquired resistance to RAF inhibitor combinations, it is striking that a different molecular mechanism of resistance was identified in each analysis, including KRAS mutation, KRAS amplification, BRAF amplification, and MEK1 mutation. The diversity of potential molecular alterations leading to acquired resistance suggests that therapeutic approaches targeting individual resistance alterations may be difficult to implement as a generalizable strategy to overcome resistance. However, the common thread among these diverse molecular alterations is that they converge on MAPK pathway reactivation as a basic mechanism for promoting resistance. Thus, it may be possible to find a key convergent signaling node that can effectively target resistance signals produced by many of these specific alterations. We found that an ERK inhibitor retained the ability to suppress MAPK signaling and could overcome each of the identified acquired resistance mechanisms. It has been previously demonstrated by our group and others that resistance to MEK inhibitors can be promoted by molecular alterations that activate the MAPK pathway.
pathway upstream of MEK, including RAS mutation or amplification and BRAF amplification, likely by leading to increased pathway flux and MEK hyperactivation that can overcome the effects of MEK inhibitors (7, 24). However, in many of these cases of upstream MAPK pathway alterations, ERK inhibitors maintain their ability to suppress the MAPK pathway, as we have observed here (17, 18). Taken together, these findings suggest that ERK inhibitors, which are currently in early-phase clinical trials, could be important components of future therapeutic strategies for BRAF-mutant colorectal cancer, either alone or in combination with RAF and EGFR inhibitors. Further studies defining the common mechanisms of clinical acquired resistance to RAF inhibitor combinations in patients with BRAF-mutant colorectal cancer will be critical to improving therapies for this lethal subtype of colorectal cancer.

**METHODS**

Detailed methods are included in Supplementary Materials.

**Patient Samples, Cell Lines, and Reagents**

Patient tumor specimens and normal blood were obtained from patients treated at the Massachusetts General Hospital under Institutional Review Board–approved studies. All patients provided written, informed consent, and studies were conducted in accordance with the Declaration of Helsinki.

VACO432 and COLO-320DM cells were obtained from the Massachusetts General Hospital Center for Molecular Therapeutics, which performs routine cell line authentication testing by SNP and short tandem repeat analysis, and were passaged less than 6 months following receipt. Cells were grown in DMEM/F12 (GIBCO) with 10% FBS and assayed in ACL-4 media (GIBCO) with 10% FBS and assayed in ACL-4 with 5% FBS. Cetuximab and panitumab were obtained from the Massachusetts General Hospital Pharmacy and diluted in PBS. Chemicals were dissolved in DMSO (Sigma), and Vx-11e (ChemieTek).

**CT Scans and RECIST Measurements**

Spiral CT scans were obtained using standard procedures in the Department of Radiology at the Massachusetts General Hospital as part of the routine clinical care of these patients. RECIST measurements were performed by radiologists in the Tumor Imaging Metrics Core at the Dana-Farber/Harvard Cancer Center using standard methods. CT images corresponding to specific RECIST target lesions were defined as the Tumor Imaging Metrics Core were obtained for display.

**WES and RNA-seq**

WES of matched pretreatment and postprogression biopsies and normal blood was performed as previously described (16, 21, 25). All BAM files were deposited in the database of Genotypes and Phenotypes (dbGaP), accession number phs00803.v1.p1. Detailed methods for RNA-seq from formalin-fixed paraffin-embedded tissue are included in Supplementary Materials.

**Western Blot Analysis and Antibodies**

Western blotting was performed using standard methods. After treatment with the indicated drugs, cells were washed with cold PBS and lysed in the following lysis buffer: 20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 10 mmol/L β-glycophosphate, 1 mmol/L sodium vanadate, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 4 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 16,000 × g for 5 minutes at 4°C. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Scientific). Proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham). Immunoblotting was performed per antibody manufacturer’s specifications. Antibodies for pMEK, pCRAF, ARAF, and BRAF (Cell Signaling Technology) were used at 1:1,000 dilution; pERK (Cell Signaling Technology) was used at 1:2,000 dilution; pRSK (Millipore) was used at 1:10,000 dilution; KRAS (Santa Cruz Biotechnology) was used at 1:500 dilution; GAPDH (Millipore) was used at 1:1,000 dilution. Protein detection on Western blot analyses was performed using SuperSignal chemiluminescence (Thermo Scientific).

**FISH**

FISH for BRAF was performed as described previously (7). FISH for KRAS was performed in the Massachusetts General Hospital Molecular Pathology Clinical Laboratory using a Clinical Laboratory Improvement Amendments (CLIA)–approved clinical assay.

**Generation of Resistant Cell Lines**

VACO432 cells were seeded at approximately 70% confluence in 10-cm plates in DMEM/F12 with 5% FBS and were treated continuously with either 3 μmol/L vemurafenib with 10 μg/mL cetuximab (VACO-RE cells) or 3 μmol/L vemurafenib with 1 μmol/L selumetinib (VACO-REM cells) until cells capable of proliferating efficiently in the presence of drug were derived. Cells were maintained in fresh drug-containing media changed every 72 to 96 hours throughout.

**Disclosure of Potential Conflicts of Interest**

E.M Van Allen is a consultant/advisory board member for Syapse and Roche Ventana. N. Wagle has ownership interest (including patents) in Foundation Medicine and is a consultant/advisory board member for the same. D. Dias-Santagata is a consultant/advisory board member for BioReference Laboratories. J.A. Engelman reports receiving commercial research support from AstraZeneca and Novartis and is a consultant/advisory board member for Roche, Genentech, GSK, AstraZeneca, and Novartis. L.A Garraway reports receiving a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member for Novartis and Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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