RESEARCH BRIEF

EGFR-Mediated Reactivation of MAPK Signaling Contributes to Insensitivity of BRAF-Mutant Colorectal Cancers to RAF Inhibition with Vemurafenib

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ABSTRACT

BRAF mutations occur in 10% to 15% of colorectal cancers and confer adverse outcome in the metastatic setting. Although RAF inhibitors such as vemurafenib (PLX4032) have proven effective in the treatment of BRAF-mutant melanoma, they are surprisingly ineffective in BRAF-mutant colorectal cancers, and the reason for this disparity remains unclear. Compared with BRAF-mutant melanoma cells, BRAF-mutant colorectal cancer cells were less sensitive to vemurafenib, and phospho-extracellular signal-regulated kinase (P-ERK) suppression was not sustained in response to treatment. Although transient inhibition of P-ERK by vemurafenib was observed in colorectal cancer, rapid ERK reactivation occurred through epidermal growth factor receptor (EGFR)-mediated activation of RAS and CRAF. BRAF-mutant colorectal cancers expressed greater levels of phospho-EGFR than BRAF-mutant melanomas, suggesting that colorectal cancers are specifically poised for EGFR-mediated resistance. Combined RAF and EGFR inhibition blocked reactivation of mitogen-activated protein kinase (MAPK) signaling in BRAF-mutant colorectal cancer cells and markedly improved efficacy in vitro and in vivo. These findings support the evaluation of combined RAF and EGFR inhibition in patients with BRAF-mutant colorectal cancer.

SIGNIFICANCE: BRAF valine 600 (V600) mutations occur in 10% to 15% of colorectal cancers, yet these tumors show a surprisingly low clinical response rate (~5%) to selective RAF inhibitors such as vemurafenib, which have produced dramatic response rates (60%-80%) in melanomas harboring the identical BRAF V600 mutation. We found that EGFR-mediated MAPK pathway reactivation leads to resistance to vemurafenib in BRAF-mutant colorectal cancers and that combined RAF and EGFR inhibition can lead to sustained MAPK pathway suppression and improved efficacy in vitro and in tumor xenografts. Cancer Discovery; 2(3); 227–35. ©2012 AACR.

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INTRODUCTION

Mutations in valine 600 (V600) of the BRAF oncogene occur in approximately 7% of all human cancers, including 10% to 15% of colorectal cancers and 50% to 60% of melanomas (1). BRAF belongs to the RAF family of kinases, which also includes ARAF and CRAF. RAF kinases normally function to activate the mitogen-activated protein kinase (MAPK) signaling pathway in response to signals from activated, GTP-bound RAS. RAF kinases phosphorylate and activate mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) kinases (MEK1 and MEK2), which in turn phosphorylate and activate ERK kinases (ERK1 and ERK2). ERK kinases phosphorylate a number of cellular substrates with key roles in cell proliferation and survival (2, 3). BRAF V600 mutations lead to constitutive BRAF kinase activity, phosphorylation of MEK and ERK kinases, and sustained MAPK pathway signaling.

In colorectal cancer, BRAF mutations are associated with adverse clinical outcome. Indeed, patients with metastatic colorectal cancer harboring BRAF V600 mutations exhibit an approximately 70% increase in mortality when compared with patients who have wild-type BRAF (4, 5). Furthermore, some investigators (6) have suggested that the presence of BRAF mutation predicts a lack of response to monoclonal antibodies against the epidermal growth factor receptor (EGFR), such as cetuximab. Therefore, novel therapeutic strategies for patients with BRAF-mutant colorectal cancers are critically needed.

Recently, the selective RAF inhibitor vemurafenib (PLX4032) was approved by the U.S. Food and Drug Administration for the treatment of metastatic melanomas harboring BRAF V600 mutations. Although RAF inhibitors such as vemurafenib have produced impressive response rates of approximately 60% to 80% in patients with BRAF-mutant melanoma (7, 8), vemurafenib demonstrated disappointing results in patients with BRAF-mutant colorectal cancer, producing only a single partial response (overall response rate of ~5%) in 19 evaluable patients (9). The reason for the difference in efficacy of vemurafenib between BRAF-mutant colorectal cancers and melanomas remains unclear. However, elucidating the mechanism of vemurafenib resistance in BRAF-mutant colorectal cancer may lead to new therapeutic strategies for this lethal subtype of colorectal cancer.

Here, we evaluated BRAF colorectal cancer and melanoma cell lines harboring BRAF V600 mutations for differences in sensitivity and signal transduction response to RAF inhibition. We found that rapid EGFR-mediated reactivation of the MAPK pathway contributes to the relative insensitivity of BRAF-mutant colorectal cancer cells to vemurafenib. We also observed that concomitant inhibition of RAF and EGFR in BRAF-mutant colorectal cancers leads to sustained suppression of MAPK signaling and to markedly increased therapeutic efficacy in vitro and in tumor xenografts. Together, our results suggest that combined RAF and EGFR inhibition may be a promising therapeutic strategy for patients with BRAF-mutant colorectal cancer.

RESULTS

To explore the difference in sensitivity to RAF inhibition between BRAF-mutant colorectal cancer and BRAF-mutant melanomas, we evaluated the effects of vemurafenib treatment on colorectal cancer and melanoma cell lines that harbor BRAF V600 mutations (Supplementary Table S1). Mirroring the disparity in clinical responsiveness to vemurafenib of BRAF-mutant colorectal cancer and melanoma, colorectal cancer cell lines demonstrated decreased sensitivity to vemurafenib in vitro (Fig. 1A). Vemurafenib led to a decrease in viable cell numbers relative to pretreatment starting cell number in BRAF-mutant melanoma cell lines. Conversely, although vemurafenib slowed the growth of BRAF-mutant colorectal cancer cells relative to untreated controls, treatment with vemurafenib failed to decrease cell numbers compared with the pretreatment starting cell number in the BRAF-mutant colorectal cancer cell lines.

Consistent with these findings, vemurafenib led to sustained suppression of phospho-ERK (P-ERK) in all melanoma cell lines (Fig. 1B and C). In contrast, vemurafenib treatment transiently suppressed P-ERK in colorectal cancer cell lines, but reaccumulation of P-ERK (to ~25%–50% of initial levels) was observed by 24 hours, indicating reactivation of the MAPK pathway. This incomplete suppression of P-ERK may underlie the relative insensitivity of BRAF-mutant colorectal cancer cells to vemurafenib because in a recent study, other investigators demonstrated that near-complete inhibition of P-ERK is required for tumor responses to vemurafenib in BRAF-mutant melanomas (10).

The rebound in P-ERK after treatment of BRAF-mutant colorectal cancer cells with vemurafenib was associated with the induction of CRAF phosphorylation at S338, indicative of activation of the CRAF kinase (Fig. 1B). The rebound in P-ERK after RAF inhibition could still be blocked by the addition of the MEK inhibitor AZD6244 (selumetinib), indicating that P-ERK reaccumulation was still MEK-dependent (Supplementary Fig. S1). Taken together, these results suggest that lack of sustained MAPK pathway inhibition may underlie the decreased sensitivity of BRAF-mutant colorectal cancer to vemurafenib.

Because CRAF phosphorylation was induced by vemurafenib in BRAF-mutant colorectal cancer cells, we investigated whether activation of RAS could account for the reactivation of MAPK signaling observed after treatment with vemurafenib. Not only can RAS activate CRAF directly, but activated RAS also can induce transactivation of BRAF-CRAF heterodimers in the presence of RAF inhibitors such as vemurafenib, leading to paradoxical activation of ERK (11–13). Consistent with this hypothesis, we found that the absolute levels of activated GTP-bound RAS were far greater after treatment with vemurafenib in BRAF-mutant colorectal cancer compared with melanoma cell lines (Fig. 2A).

To determine whether activation of receptor tyrosine kinase (RTK) signaling might account for the observed differences in RAS activation, we evaluated global RTK phosphorylation in BRAF-mutant colorectal cancer and melanoma cell lines in the presence or absence of vemurafenib by using phospho-RTK arrays. Interestingly, we found that RTK phosphorylation was universally low in BRAF-mutant melanoma cells before and after treatment with vemurafenib (Fig. 2B). By contrast, BRAF-mutant colorectal cancer cells displayed high basal levels of several phosphorylated RTKs, including EGFR, HER2, MET, and insulin-like growth factor 1 receptor (IGF1R).
Figure 1. Incomplete suppression of P-ERK in BRAF-mutant colorectal cancers (CRC) is associated with decreased sensitivity to vemurafenib. A, BRAF-mutant melanoma and colorectal cancer cell lines were treated with (VEM) or without (CON) 3 μM vemurafenib for 72 hours, and viable cell titer was determined with the Cell TiterGlo assay. Values represent the change in viable cell titer relative to the starting cell titer immediately before treatment. B, BRAF-mutant cell lines from (A) were treated with 3 μM vemurafenib for the indicated times, and lysates were probed with the indicated antibodies. C, chemiluminescent quantifications of normalized P-ERK levels from Western blots as in (B) are illustrated. Values represent mean of 3 independent experiments.
Notably, with the exception of IGF1R, treatment with vemurafenib did not induce phosphorylation of any of these RTKs. Elevated levels of phospho-EGFR (P-EGFR), phospho-HER2 (P-HER2), phospho-MET (P-MET), and phospho-IGF1R (P-IGF1R) in BRAF-mutant colorectal cancer cells were confirmed by Western blot (Fig. 2C). Protein expression levels of EGFR and MET also were elevated in colorectal cancer cells relative to melanoma cells. However, only total and phosphorylated EGFR levels were elevated specifically in all of the BRAF-mutant colorectal cancer cell lines.

To determine whether a specific RTK might predominantly lead to activation of RAS and reactivation of MAPK signaling in BRAF-mutant colorectal cancer cells treated with vemurafenib, BRAF-mutant colorectal cancer cells were treated with small-molecule kinase inhibitors of the aforementioned RTKs in the presence or absence of vemurafenib. Inhibition of IGF1R (with NVP-AEW541, a selective small-molecule inhibitor of IGF1R; ref. 14) or MET (with crizotinib) failed to maintain P-ERK suppression in the presence of vemurafenib (Fig. 3A), even though target RTK inhibition was achieved at the inhibitor concentration used (Supplementary Fig. S2A–D). However, treatment with the EGFR inhibitor gefitinib or with the dual EGFR/HER2 inhibitor lapatinib led to more complete suppression of P-ERK upon vemurafenib treatment.

Because similar suppression of P-ERK in the presence of vemurafenib was observed with gefitinib and lapatinib, it is likely that EGFR, and not HER2, is the predominant mediator of MAPK reactivation upon RAF inhibition (although a potential role for HER2 in BRAF-mutant colorectal cancer is still possible). More complete suppression of P-ERK also was observed in cells treated with vemurafenib and the EGFR inhibitor erlotinib and in cells transfected with siRNA directed against EGFR, supporting the importance of EGFR in the reactivation of ERK signaling (Supplementary Fig. S2D and E).

Inhibition of EGFR with gefitinib abrogated the induction of activated RAS (RAS-GTP) by vemurafenib in BRAF-mutant colorectal cancer cell lines (Fig. 3B), supporting a role for EGFR as the major activator of RAS in these cells. Accordingly, treatment with gefitinib also abrogated the induction of P-CRAF in vemurafenib-treated BRAF-mutant colorectal cancer cells (Fig. 3C). Interestingly, P-EGFR levels did not clearly increase after treatment with vemurafenib at any time point tested between 0 and 48 hours, although MAPK activity appeared to recover as early as 3 to 6 hours after treatment with vemurafenib (Fig. 3C, Supplementary Fig. S1, and Supplementary Fig. S3). These results suggest that EGFR activation does not increase upon treatment with vemurafenib but that EGFR is able to more effectively engage downstream signaling pathways after treatment with vemurafenib.

Consistent with the sustained P-ERK suppression achieved in BRAF-mutant colorectal cancer cells treated with gefitinib and vemurafenib, improved in vitro efficacy was observed with this inhibitor combination (Fig. 3D). Greater inhibition of viable cell number compared with vemurafenib alone was observed in all BRAF-mutant cell lines, and all but one cell line showed an absolute decrease in viable cell number relative to the pretreatment starting cell number. The decrease in cell viability...
sensitivity to MAPK inhibition (15). Notably, inhibition of EGFR did not block P-AKT induction by vemurafenib (Supplementary Fig. S5), despite the profound effect of this combination on cell viability. Previous work from our laboratory has implicated IGF1R as the predominant regulator of PI3K signaling in colorectal cancer, including BRAF-mutant colorectal cancer (16). Accordingly, we found that induction of P-AKT by vemurafenib was associated with an increase in P-IGF1R and that cotreatment with a small-molecule inhibitor of IGF1R could abrogate induction of P-AKT (Supplementary Fig. S5).

IGF1R inhibition blocked the induction of P-AKT completely (>90%) in WiDr cells and by approximately 50% in HT-29 cells. However, although IGF1R inhibition was achieved with combined vemurafenib and gefitinib was significantly greater than that achieved with vemurafenib in combination with other inhibitors (NVP-AEW541 and crizotinib) that did not lead to improved suppression of P-ERK (Supplementary Fig. S4 and Fig. 3A). Taken together, these data suggest that EGFR-mediated RAS activation leads to reactivation of MAPK signaling in many BRAF-mutant colorectal cancers and that combined inhibition of RAF and EGFR may lead to improved efficacy in these cancers.

Vemurafenib also led to induction of P-AKT, an important signaling component of the PI3K pathway (Supplementary Fig. S5). Induction of PI3K-AKT pathway signaling has previously been associated with decreased sensitivity to MAPK inhibition (15). Notably, inhibition of EGFR did not block P-AKT induction by vemurafenib (Supplementary Fig. S5), despite the profound effect of this combination on cell viability. Previous work from our laboratory has implicated IGF1R as the predominant regulator of PI3K signaling in colorectal cancer, including BRAF-mutant colorectal cancer (16). Accordingly, we found that induction of P-AKT by vemurafenib was associated with an increase in P-IGF1R and that cotreatment with a small-molecule inhibitor of IGF1R could abrogate induction of P-AKT (Supplementary Fig. S5).

IGF1R inhibition blocked the induction of P-AKT completely (>90%) in WiDr cells and by approximately 50% in HT-29 cells. However, although IGF1R inhibition
limited the induction of P-AKT by vemurafenib, this combination was still less effective than vemurafenib and gefitinib (Supplementary Fig. S4). The failure of IGF1R inhibition to improve suppression of P-ERK by vemurafenib (Fig. 3A and Supplementary Fig. S5) likely accounts for the increased sensitivity of BRAF-mutant colorectal cancer cells to combined EGFR/RAF inhibition than to combined IGF1R/RAF inhibition and supports the notion that these BRAF-mutant cancer cells are highly dependent on MEK-ERK signaling.

Given the sustained suppression of P-ERK signaling and improved in vitro efficacy of combined RAF and EGFR inhibition, we next tested whether this inhibitor combination strategy was effective in vivo by using BRAF-mutant colorectal cancer xenografts. Relative to vehicle-treated controls, treatment with vemurafenib alone (at a dose previously determined to be optimal for in vivo mouse studies; ref. 17) or with the EGFR inhibitor erlotinib alone led to only modest inhibition of tumor growth in HT-29 xenografts and no significant tumor inhibition in WiDr xenografts (Fig. 4A). However, the combination of vemurafenib and erlotinib led to dramatic tumor inhibition and caused regressions in most tumors (Fig. 4A and B). Mice tolerated the combined treatment well (Supplementary Fig. S6). Combined treatment with vemurafenib and erlotinib also led to improved inhibition of P-ERK relative to either treatment alone and to improved inhibition of tumor cell proliferation as assessed by Ki67 staining (Fig. 4C and Supplementary Fig. S7A and B). These results support the notion that combined inhibition of RAF and EGFR may be a promising therapeutic strategy for BRAF-mutant colorectal cancer.

To explore whether EGFR might play a role in the insensitivity of human BRAF-mutant colorectal cancers to vemurafenib, we evaluated P-EGFR levels in BRAF-mutant human colorectal cancers. P-EGFR was detected in all cases of BRAF-mutant colorectal cancer examined (Fig. 4D). When compared with BRAF-mutant melanomas, BRAF-mutant colorectal cancers exhibited significantly greater levels of P-EGFR (Fig. 4D), which is consistent with our findings in cell lines (Fig. 2C) and supports the idea that human BRAF-mutant colorectal cancers may be more poised to exhibit EGFR-mediated resistance than BRAF-mutant melanomas. Interestingly, 60% of BRAF-mutant colorectal cancer cases (n = 10) expressed particularly high levels of P-EGFR (scored as 2 or 3, as described in the Methods) compared with only 18% of melanoma cases with similarly elevated expression (n = 11; P < 0.05), raising the possibility that levels of P-EGFR might predict which BRAF-mutant colorectal cancers are most likely to develop EGFR-mediated resistance to RAF inhibition.

DISCUSSION

Although selective RAF inhibitors such as vemurafenib have produced dramatic responses in BRAF V600E-mutant melanomas, colorectal cancers harboring identical BRAF V600 mutations have failed to respond (7–9). Here, we present evidence that EGFR-mediated reactivation of MAPK signaling in BRAF-mutant colorectal cancer leads to incomplete P-ERK suppression to vemurafenib, resulting in reduced sensitivity. This resistance mechanism appears to involve activation of RAS by EGFR, leading to greater levels of activated RAS and P-CRAF induction in BRAF-mutant colorectal cancers than in BRAF-mutant melanomas. The authors of recent studies have shown that activated RAS can cause MAPK pathway activation through direct activation of CRAF or by the transactivation of BRAF–CRAF heterodimers in the presence of vemurafenib (11–13), or possibly through a combination of both mechanisms. Indeed, introduction of an activated RAS mutant into HT-29 cells led to sustained P-ERK levels and resistance to vemurafenib (11). We found that inhibition of EGFR abrogated RAS activation, P-CRAF induction, and P-ERK reactivation upon treatment with vemurafenib in BRAF-mutant colorectal cancer cells (Fig. 3A–C), suggesting that vemurafenib can produce sustained inhibition of mutant BRAF activity and suppression of ERK phosphorylation in the absence of EGFR-mediated feedback signals. Notably, we found that the sustained suppression of P-ERK achieved by combined RAF and EGFR inhibition leads to increased sensitivity in vitro and to tumor regressions in vivo (Fig. 3D and Fig. 4A and B). These findings suggest that BRAF-mutant colorectal cancers, like their melanoma counterparts, retain a strong dependency on MAPK signaling and that tumor responses are possible if the MAPK pathway is adequately inhibited in these cancers.

Interestingly, although EGFR appeared to mediate reactivation of MAPK signaling in response to vemurafenib, we did not observe evidence of increased EGFR activation per se after treatment with vemurafenib, as might be expected in a classic feedback loop. Indeed, P-EGFR levels did not increase after vemurafenib treatment at any time point tested between 0 and 48 hours, although MAPK activity appeared to recover as early as 3 to 6 hours after treatment with vemurafenib (Fig. 3C, Supplementary Fig. S1, and Supplementary Fig. S3). In fact, if anything, a slight decrease in P-EGFR and total EGFR levels was observed at later time points. These findings suggest that EGFR is active in BRAF-mutant colorectal cancer cells before vemurafenib treatment but that EGFR transmits its signal to activate RAS and CRAF only upon vemurafenib treatment (Supplementary Fig. S8). One possible explanation for this observation may involve Sprouty proteins, which are important MAPK pathway feedback mediators that are transcribed in an ERK-dependent manner. Sprouty proteins can block RTK-mediated activation of RAS (18). Consistent with this hypothesis, we observed that Sprouty4 (Spry4) levels decreased after treatment with vemurafenib, and this decrease coincided with induction of P-CRAF and P-ERK (Supplementary Fig. S9). Still, further studies are necessary to determine whether Sprouty proteins are involved in this de-repression of EGFR-dependent activation of downstream signaling.

BRAF-mutant colorectal cancer cell lines expressed greater levels of EGFR and P-EGFR than BRAF-mutant melanoma cell lines, and human BRAF-mutant colorectal cancers exhibited significantly greater levels of P-EGFR than BRAF-mutant melanomas (Figs. 2C and 4D). These observations may explain why BRAF-mutant colorectal cancers are more susceptible to EGFR-mediated RAF inhibitor resistance through
EGFR Mediates RAF Inhibitor Resistance in BRAF-Mutant Colorectal Cancer

**A**

**Figure 4.** Combined RAF and EGFR inhibition leads to improved in vivo efficacy in BRAF-mutant colorectal cancer. **A,** BRAF-mutant colorectal cancer xenografts derived from HT-29 and WiDr cells were treated with vehicle only (CON), vemurafenib only (VEM, 75 mpk twice daily), erlotinib (ERL, 100 mpk daily), or both inhibitors (VEM/ERL) in combination for 21 days. Average percent change in tumor volume relative to initial tumor volume is shown. Error bars represent SEM. **P** < 0.001 for combined vemurafenib/erlotinib vs. all other treatment groups. **B,** waterfall plots showing the percent change in volume (relative to initial tumor volume) for the individual tumors in each treatment group. **C,** tumor tissue from HT-29 xenografts treated for 3 days as indicated was evaluated by IHC for P-ERK and a marker of cell proliferation (Ki67). Tumors were harvested 4 hours after dosing on day 3. **D,** levels of P-EGFR were assessed in human BRAF-mutant colorectal cancers (CRC) and melanomas by IHC. Representative examples are shown. Colorectal cancer cases with the lowest (C3) and highest (C7) P-EGFR levels are shown. A total of 60% of BRAF-mutant colorectal cancers (n = 10) exhibited high levels of P-EGFR, whereas only 18% of BRAF-mutant melanomas (n = 11) exhibited high levels of P-EGFR (P < 0.05).
incomplete MAPK suppression. Interestingly, although BRAF-mutant melanoma cells had globally low levels of phosphorylated RTKs (perhaps explaining their exquisite sensitivity to single-agent RAF inhibitors), BRAF-mutant colorectal cancer cells exhibited high levels of several phosphorylated RTKs. This finding raises the possibility that other RTKs in addition to EGFR (e.g., HER2, MET, IGF1R) could mediate resistance to RAF inhibitors through activation of RAS and the MAPK pathway.

In our colorectal cancer cell line models we observed that EGFR appeared to exert dominant control over RAS and the MAPK pathway, despite the presence of these additional phosphorylated RTKs (Fig. 3A–C). However, it remains possible that some BRAF-mutant colorectal cancers may depend on RTKs other than EGFR. Interestingly, although we detected the presence of P-EGFR in all cases of BRAF-mutant colorectal cancer evaluated, we observed that a subset of these cancers (60%) exhibited particularly high P-EGFR levels (Fig. 4D). Future studies will determine whether P-EGFR levels can predict which patients might benefit most from combined RAF/EGFR inhibition and which might benefit from an alternative approach (e.g., combined RAF/MEK inhibition (Supplementary Fig. S1), currently in clinical trials for BRAF-mutant colorectal cancer; ref. 19). In summary, the improved suppression of MAPK signaling and the substantial tumor regressions observed in our xenograft studies support the evaluation of combined RAF/EGFR inhibition in clinical trials for patients with BRAF-mutant colorectal cancer.

METHODS

Detailed methods are included in the Supplementary Methods.

Cell Lines, Reagents, and Patient Samples

All cell lines were grown in DMEM/F12 ( Gibco) with 10% FBS and assayed in DMEM/F12 with 5% FBS; cell lines were obtained from the Massachusetts General Hospital Center for Molecular Therapeutics, which performs routine cell line authentication testing by single-nucleotide polymorphism and short tandem repeat analysis. Genotype data were obtained from the Sanger Cancer Genome Project (www.sanger.ac.uk/genetics/CGP). Chemical inhibitors from the following sources were dissolved in DMSO for in vitro studies: vemurafenib (Active Biochem); gefitinib, erlotinib, and lapatinib (LC Laboratories); NVP-AEW541 (Selleck Chemicals); and crizotinib (ChemieTek); and AZD6244 (Otava Chemicals). Human tumor specimens were obtained from the Massachusetts General Hospital under Institutional Review Board-approved studies. All patients provided written, informed consent. BRAF mutation status was determined by the Massachusetts General Hospital Clinical Laboratory and Department of Pathology.

Xenograft Studies

HT-29 or WiDr cells were injected (5 × 10⁶ cells per injection) into the flanks of male athymic nude mice (Charles River Laboratories). Once tumors reached an average volume of approximately 100 to 200 mm³, mice were randomized into treatment arms and tumor volume was assessed by caliper measurements during a 21-day period. For pharmacodynamic studies, tumor tissue was harvested and formalin-fixed 4 hours after the morning doses of drug on the third day of treatment. Vemurafenib and erlotinib for in vivo studies were obtained from the Massachusetts General Hospital Pharmacy. Vemurafenib was formulated in 5% dimethyl sulfoxide, 1% methylcellulose, and dosed at 75 mg/kg twice daily by oral gavage. Erlotinib was formulated in polysorbate and dosed at 100 mg/kg daily. Animal care and treatment was performed in accordance with institutional guidelines.

Immunohistochemistry

Immunohistochemistry (IHC) on formalin-fixed paraffin-embedded tissue was performed for P-ERK as previously described (20). IHC for P-EGFR was performed with the use of P-EGFR Y1068 antibody (Cell Signaling 3777, 1:800 dilution in SignalStat Antibody Diluent) according to the manufacturer’s protocol. IHC for Ki67 was performed with the use of Ki67 antibody (Novocastra/Leica NCL-Ki67p at 1:1000 dilution in PBS/3% bovine serum albumin) and developed with the use of Dako Envision Plus System/HRP (DAB). P-EGFR IHC intensity scoring of all human colorectal cancer and melanoma specimens was performed by the same pathologists (A. Piris and M. Nishino). Intensities of 0 (no staining), 1 (low staining), 2 (intermediate staining), and 3 (high staining) were used with P-EGFR staining in normal colonic crypts as a standard for a score of 3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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EGFR Mediates RAF Inhibitor Resistance in BRAF-Mutant Colorectal Cancer


EGFR-Mediated Reactivation of MAPK Signaling Contributes to Insensitivity of \textit{BRAF}-Mutant Colorectal Cancers to RAF Inhibition with Vemurafenib

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