Reactivation of Mitogen-activated Protein Kinase (MAPK) Pathway by FGF Receptor 3 (FGFR3)/Ras Mediates Resistance to Vemurafenib in Human B-RAF V600E Mutant Melanoma

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Background: B-RAF V600E melanomas rapidly develop resistance to B-RAF inhibitors in the clinic.
Results: FGFR3/Ras signaling is elevated and induces resistance to vemurafenib in vemurafenib-resistant cells.
Conclusion: FGFR3/Ras confers resistance to B-RAF inhibition via MAPK pathway reactivation.
Significance: A novel mechanism of resistance to B-RAF inhibitors is described and potential therapeutic strategies are suggested.

Oncogenic B-RAF V600E mutation is found in 50% of melanomas and drives MEK/ERK pathway and cancer progression. Recently, a selective B-RAF inhibitor, vemurafenib (PLX4032), received clinical approval for treatment of melanoma with B-RAF V600E mutation. However, patients on vemurafenib eventually develop resistance to the drug and demonstrate tumor progression within an average of 7 months. Recent reports indicated that multiple complex and context-dependent mechanisms may confer resistance to B-RAF inhibition. In the study described herein, we generated B-RAF V600E melanoma cell lines of acquired-resistance to vemurafenib, and investigated the underlying mechanism(s) of resistance. Biochemical analysis revealed that MEK/ERK reactivation through Ras is the key resistance mechanism in these cells. Further analysis of total gene expression by microarray confirmed a significant increase of Ras and RTK gene signatures in the vemurafenib-resistant cells. Mechanistically, we found that the enhanced activation of fibroblast growth factor receptor 3 (FGFR3) is linked to Ras and MAPK activation, therefore conferring vemurafenib resistance. Pharmacological or genetic inhibition of the FGFR3/Ras axis restored the sensitivity of vemurafenib-resistant cells to vemurafenib. Additionally, activation of FGFR3 sufficiently reactivated Ras/MAPK signaling and conferred resistance to vemurafenib in the parental B-RAF V600E melanoma cells. Finally, we demonstrated that vemurafenib-resistant cells maintain their addiction to the MAPK pathway, and inhibition of MEK or pan-RAF activities is an effective therapeutic strategy to overcome acquired-resistance to vemurafenib. Together, we describe a novel FGFR3/Ras mediated mechanism for acquired-resistance to B-RAF inhibition. Our results have implications for the development of new therapeutic strategies to improve the outcome of patients with B-RAF V600E melanoma.

Melanoma is the sixth most common cancer in the United States, and the incidence continues to rise across the world (1). Metastatic melanoma has a poor prognosis with a five year survival rate of less than 20% (2). The Ras-RAF-MEK-ERK MAP kinase signaling plays an important role in melanoma etiology and progression, and is considered a key target for anti-melanoma therapies (3, 4). Approximately 50% of melanomas harbor an activating B-RAF V600E mutation that leads to constitutive activation of the B-RAF kinase and downstream MAPK signaling (4, 5). B-RAF V600E melanoma cells are addicted to the constitutive activation of the MAPK signaling and are highly sensitive to B-RAF inhibition (6). As a result, B-RAF is considered an attractive target for treatment of melanoma.

Vemurafenib/PLX4032 and PLX4720 (preclinical analog of PLX4032) are potent small molecule inhibitors of mutant B-RAF V600E (7–9). Vemurafenib was recently approved by United States Food and Drug Administration for treatment of metastatic and unresectable melanomas that carry an activating B-RAF V600E mutation. In recent clinical studies in patients with metastatic melanomas carrying mutant B-RAF, single agent vemurafenib exhibited robust efficacy with a response rate of 50%, and improved overall survival as compared with the chemotherapeutic agent dacarbazine (10, 11). Although these patient responses were encouraging, they were relatively short-lived. Almost all of the patients who initially responded to vemurafenib therapy developed drug resistance and eventually relapsed within an average of 7 months (10). Therefore, similar to many other targeted therapies, the acquired resistance to B-RAF inhibition presents a significant therapeutic challenge to long-term survival benefit in this patient population.

To improve the clinical benefit of B-RAF inhibitors, it is urgent and critical to identify the mechanisms which render mutant B-RAF-expressing melanoma cells resistant to B-RAF inhibition. Recent studies have indicated that reactivation of the MAPK pathway is an important mechanism of resistance to B-RAF inhibition. Resistant mechanisms primarily involve reactivation of ERK signaling via bypass mechanisms that are
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either Ras/RAF dependent, such as Ras activation (12, 13) or C-RAF up-regulation (14, 15), or likely Ras/RAF independent (Tpl2/COT overexpression) (14). Recently an activating mutation in MEK was reported in the vemurafenib-resistant tumor in one patient (16). Thus, multiple mechanisms could attenuate the effect of B-RAF inhibition on MAPK signaling in B-RAF mutant melanomas. Recent studies have also suggested that activation of MAPK-redundant signaling pathways by receptor tyrosine kinases (RTKs)\(^2\) such as IGF-1R or PDGFR\(\beta\) could play a role in acquired resistance to B-RAF inhibition (12, 17, 18).

In the study described herein, we have generated vemurafenib-resistant cell lines by chronic treatment of the human melanoma cell lines, A375 and M14 both harboring the B-RAF V600E mutation with increasing concentrations of vemurafenib or its close structural analog PLX4720. Using these acquired-resistance cell models, we describe FGFR3/Ras signaling pathway as a novel mechanism for acquired-resistance to B-RAF inhibition through MAPK pathway reactivation. Our results shed new light on the complexity of the underlying signaling pathways responsible for resistance to B-RAF inhibition and may provide strategies to overcome vemurafenib resistance in the clinic.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Transfections—A375 and M14 human melanoma cells with B-RAF V600E mutation were obtained from ATCC. All of the parental and resistant cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Scientific) supplemented with 10% fetal bovine serum (FBS, Invitrogen) only. B-RAF inhibitors, PLX4720 and vemurafenib were obtained from Axon MedChem BV. MEK inhibitor AZD6244, pan-Raf inhibitor RAF265 and FGFR inhibitor LY2874455 were synthesized by Eli Lilly and Company. siRNAs for target gene knock down were obtained from Thermo Scientific (OnTargetPlus SiRNA Pools, Dharmacon). siRNA transfections were carried out using Lipofectamine\^{TM} RNAiMAX transfection reagent (Invitrogen) as per the manufacturer’s instructions. All plasmid transient transfections were carried out using FuGENE\^{®} HD Transfection Reagent according to the manufacturer’s instructions (Promega).

Generation of B-RAF V600E Melanoma Cell Lines Resistant to B-RAF Inhibition—To generate resistant cells, we cultured A375 or M14 cells in growth medium in the presence of vehicle (0.1% DMSO) or gradually increasing concentrations of PLX4720 or vemurafenib from 0.02 to 2 \(\mu\)M through ~4 months and 30 passages to result in resistant cell lines designated as A375-R1, A375-R3, and M14-R. A375-R1 was generated by treatment with PLX4720 and, A375-R3 and M14-R cells were generated by treatment with vemurafenib.

Cell Proliferation Assay—Cells (5 \(\times\) 10\(^3\)), maintained in growth medium described above, were plated onto poly-d-lysine-coated well in 96-well plates (BD Biosciences) a day before the treatment. The cells were treated for 48–72 h, and then analyzed for viability using the CellTiterGlo Luminescent Cell Viability Assay according to the manufacturer’s instructions (Promega) and a SpectraMax plate reader (Molecular Devices). Nonlinear regression and sigmoidal dose-response curves were used to calculate the half maximal inhibitory concentration (IC\(_{50}\)) with GraphPad Prism 4 software.

Immunoblotting and Phospho-antibody Array—Western blotting analysis was performed as described previously (19). Proteins were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences). Antibodies against P-Tyr724-FGFR3 (sc-33041) and FGFR3 (c-15) were obtained from Santa Cruz Biotechnology, Inc. Antibodies against ERK1/2, phospho-ERK1/2 (9101), phospho-Akt1 (S473) (4060), phospho-FGFR (3476), A-Raf (4432), B-Raf (9434), C-Raf (9422), phospho-MEK1/2 (9154), and Akt (9272), were obtained from Cell Signaling Technology. Anti-tubulin (ab7291, AbCam), bFGF (MILipore), and anti-Ras (Upstate) antibodies were obtained from the indicated companies. To identify the relative phosphorylation levels of human RTKs, we used human RTK Signaling Antibody Array Kit (7949, Cell Signaling Technologies) for phospho-protein analysis according to the manufacturer’s instructions. Supplemental Table S2 describes the list of RTKs and the layout of the antibody array.

Ras Activation Assay—Ras activation assay was performed using Ras Activation Assay Kit from Millipore as per the manufacturer’s instructions. Briefly, 500 \(\mu\)l of cell lysates (protein concentration: 4 \(\mu\)g/\(\mu\)l) were incubated with 60 \(\mu\)l of Raf Ras-binding domain (RBD)-conjugated agarose beads for 1 h at 4 °C. Lysates loaded with GDP or with GTP\(\gamma\)S (non hydrolysable form of GTP) for 30 min were used as negative and positive controls, respectively. After three times washing in 1 × PBS, the beads were then boiled in reducing sample buffer. Levels of active Ras (Ras-GTP) were assessed by Western blotting using monoclonal anti-Ras antibody (1 \(\mu\)g/ml, Upstate) via Odyssey Infrared Imaging System as described above.

Mutational and Sequencing Analysis—Genomic DNA was purified from A375 and A375-R1 cells using Wizard Genomic DNA purification kit (Promega) as per manufacturer’s instructions. Genotyping and bidirectional DNA sequencing were performed by Agencourt Biosciences (Beverly, MA).

Microarray Study—Total RNA was isolated from five independent cultures of A375 and A375-R1 cells using RNAeasy Kit from Qiagen as per manufacturer’s instructions. Affymetrix gene expression profiling was performed by Asuragen Inc. (Austin, TX). Briefly, biotin-labeled targets (cRNA) were prepared using a MessageAmp\(^{\text{TM}}\) II-based protocol (Ambion Inc., Austin, TX). The cRNA yields were quantified by UV spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labeled cRNA was fragmented and used for HG-U133 Plus 2.0 microarray hybridization and washing, according to the manufacturer’s protocol (Affymetrix, Foster City, CA). Then the Affymetrix MAS5 algorithm and quantile normalization across samples were applied, followed by log2 transformation and mean-centered standardization. A total of 13678 genes that were reliably detected on at least 80% of the arrays with a signal intensity of 64 or greater were used for further analysis.
Genetic analysis of acquired resistance—Gene Set Enrichment Analysis (GSEA)—GSEA was performed as previously described (20, 21), using a total of 3272 gene sets from a Molecular Signature Data base called MolSigDB (21) (version c2.v3). The enrichment with GSEA was assessed by: (a) ranking all genes on the HG-U133PLUS2 chip with respect to the phenotype “resistant (A375-R1) versus non-resistant (A375 parental)”; (b) locating the represented members of a given gene set within the ranked genes; and (c) measuring the proximity of each gene set with respect to both ends of the ranked list using a Kolmogorov-Smirnoff (KS) non-parametric rank statistic score (with a higher score corresponding to a higher proximity); (d) comparing the observed KS score to the distribution of 1000 permuted KS scores for all gene sets. An empirical cutoff, FDR < .05, was used to define statistical significance.

Human FGF2 ELISA—Cells were cultured for 48 h in growth medium described above, and the conditioned medium samples (cell free culture supernatant) were analyzed for concentrations of human FGF2 using human FGF2 Quantikine ELISA Kit (R&D Systems). ELISA was performed as per the manufacturer’s instructions.

RESULTS

Generation of Cell Line Models of Acquired-resistance to B-RAF V600E Inhibitors—To investigate the potential mechanisms of resistance to B-RAF inhibition in melanoma, we used A375 and M14 human melanoma cell lines. These cells carry the B-RAF V600E mutation and have been previously demonstrated to be highly sensitive to B-RAF inhibition (22). After confirming the sensitivity of these cells to B-Raf inhibitors, PLX4720, and vemurafenib in cell proliferation assays, we treated and cultured these cells in the presence of either vehicle (0.1% DMSO) or gradually increasing concentrations of vemurafenib as high as 3 μM through ~4 months and 30 passages (Fig. 1A). A375-R1 was generated using PLX4720, and A375-R3 and M14-R cells were generated using vemurafenib after the publication of its chemical structure (9). As demonstrated in Fig. 1B, vehicle-treated A375 parental cells remained sensitive to vemurafenib inhibition in Cell Titer Glo assay with IC_{50} of 85 nM, while A375-R1 and A375-R3 cells were only sensitive at concentrations ~2 orders of magnitude higher (IC_{50} of 4800 nM and 3000 nM, respectively). Similarly, M14 parental cells were sensitive to vemurafenib with IC_{50} of 180 nM while M14-R cells were resistant to vemurafenib with IC_{50} of 9500 nM (Fig. 1C). The resistant cell lines were maintained in the absence of B-Raf inhibition. Interestingly, late-passage (>20) resistant cells demonstrated increased sensitivity to B-Raf inhibition when compared with early-passage (<10) resistant cells (data not shown). Therefore, all of the experiments in this study were carried out using early passage-resistant cells only.

MEK/ERK Signaling Is Reactivated and Resistant to B-RAF Inhibition in Vemurafenib-resistant Cells—We characterized the A375-R1 cell line further. We initially examined the downstream phospho-MEK and phospho-ERK activities in A375 and A375-R1 cells by immunoblotting. As revealed in Fig. 1D, the basal levels of phospho-MEK and phospho-ERK were elevated in the A375-R1 cells, while the changes in phospho-AKT levels remained sensitive to vemurafenib inhibition in Cell Titer Glo in the A375-R1 cells, while the changes in phospho-AKT levels basal levels of phospho-MEK and phospho-ERK were elevated in the A375-R1 cell line further. We initially examined the downstream phospho-MEK and phospho-ERK activities in A375 and A375-R1 cells by immunoblotting. As revealed in Fig. 1D, the basal levels of phospho-MEK and phospho-ERK were elevated in the A375-R1 cells, while the changes in phospho-AKT levels remained sensitive to vemurafenib inhibition in Cell Titer Glo in the A375-R1 cells, while the changes in phospho-AKT levels were minimal. As expected, treatment of parental cells with vemurafenib caused a robust dose dependent inhibition of MEK and ERK activities, and 0.1 μM of vemurafenib significantly reduced phospho-MEK and phospho-ERK levels. However, phospho-MEK and phospho-ERK levels remained relatively elevated in the A375-R1 cells in presence of vemurafenib as high as 3 μM (Fig. 1D). Similar to A375-R1, A375-R3 and M14-R cells also displayed sustained phospho-ERK and phospho-MEK activity in the presence of vemurafenib as high as 10 μM (supplemental Fig. S1). The continued activation of the MAPK pathway observed in the presence of vemurafenib in A375-R1 cells is believed to be a major reason of the resistance.

Mutational and Microarray Analysis of A375 and A375-R1 Cells—To determine if any activating mutation associated with MAPK pathway is responsible for MAPK reactivation and resistance in A375-R1 cells, we isolated genomic DNA and conducted mutational analysis by direct bidirectional DNA sequencing of the following genes: RAF (A-RAF, B-RAF, C-RAF), Ras (H-Ras, K-Ras, N-Ras), RTK families (EGFRs, PDGFRs, cMet, FGFRs, RET, IGF-1R, VEGFRs, etc), MEK (MEK1 and MEK2), and ERK (ERK1 and ERK2), of both A375 and A375-R1 cells as described under “Experimental Procedures.” Genotyping analysis confirmed that both A375 and A375-R1 cells have retained B-RAF V600E mutation. However, no additional mutation was observed in either cell line among the genes analyzed. To identify potential mechanisms involved in resistance of A375-R1 cells, we also performed a microarray gene expression analysis utilizing the total RNA isolated from A375 and A375-R1 cells. As shown in Fig. 2A, comparative analysis of gene expression patterns between A375 and A375-R1 cells revealed that the downstream gene signatures of oncogenic Ras and RTK signaling are among the most consistent changes, indicating that RTK/Ras signaling is potentially involved in resistance of melanoma cells to B-RAF inhibition. The Ras and RTK gene signatures were defined based on previous studies (23, 24).

Ras Is Activated in Vemurafenib-resistant Cells and Required for Resistance—RAF/MEK/ERK signaling is the key downstream effector of Ras. Reactivation of MAPK signaling (Fig. 1D) and increase of Ras gene signature by microarray study (Fig. 2A) triggered us to validate the activation status of Ras in vemurafenib-resistant cells. For this purpose, we determined the levels of active Ras in the parental A375 and vemurafenib-resistant A375-R1 and A375-R3 cells by immunoprecipitating active Ras using beads coated with GST-bound Raf binding domain (RBD) as described under “Experimental Procedures.” As shown in Fig. 2B, we observed significantly elevated levels of GTP bound Ras (Ras-GTP) in A375-R1 and A375-R3 cells when compared with parental A375 cells. We verified the efficiency of this assay by pretreating the cell lysates with GTPγS and immunoprecipitated similar levels of Ras-GTP among A375 and A375-R1 cells.

Furthermore, we investigated if active Ras is responsible for the resistance of A375-R1 cells to the B-RAF inhibition. We performed specific knockdown of total Ras using siRNA pool (supplemental Fig. S2) and evaluated its effect on cell proliferation in the presence of vemurafenib. Knockdown of Ras has no
significant effect on the sensitivity of parental cells to vemurafenib (Fig. 2C). In contrast, knockdown of Ras restored the sensitivity of resistant A375-R1 and A375-R3 cells to vemurafenib inhibition (Fig. 2C). This indicates that Ras is required for resistance to B-RAF inhibition in A375-R1 cells, although the role of individual Ras isoform has not been defined in this study.

**FGFR3 Signaling Is Elevated in Vemurafenib-resistant Cells—** Ras is generally activated in response to activation of RTKs by growth factors or activating mutation. Mutational analysis of RTKs and other components by direct DNA sequencing revealed no additional activating mutations among these genes. To identify the mechanism of Ras activation in the vemurafenib-resistant cells, we used a phospho-antibody array and analyzed the tyrosine phosphorylation levels of 28 different RTKs in parental and vemurafenib-resistant cells. Interestingly, we detected significantly elevated levels of phospho-FGFR3 in all of the three resistant cells A375-R1, M14-R, and A375-R3 cells when compared with their parental cells (Fig. 3A and supplemental Fig. S3A). To further validate the activation of FGFR3, we examined the levels of phospho-Y724 FGFR3, the active form of FGFR3 (25) in parental and resistant cells by immunoblotting. As demonstrated in Fig. 3B, increased phospho-Y724-FGFR3 levels were observed in the A375-R1 and M14-R cells. Similarly, increased phospho-FGFR3 levels were observed in A375-R3 cells (supplemental Fig. S3B). In tumors and cell lines, FGFR signaling is often enhanced by gene amplification and elevated autocrine or paracrine activation (26). Somatic gain-of-function mutations in FGFRs have also been reported in cancers such as colorectal, urothelial endometrial and melanoma (27–29). However, we did not detect any mutation in the members FGFR gene family in A375-R1 cells by DNA sequencing (data not shown). In addition, we did not detect
any significant change in the total protein levels of FGFR3 in the parental and the resistant cells by immunoblotting (Fig. 3B). However, we detected increased levels of secreted FGF2, the major ligand of FGFRs, in the growth media of A375-R1 and M14-R cells when compared with their respective parental cells by ELISA (Fig. 3C). We also measured the protein levels of bFGF by Western blot analysis and detected significant increase of bFGF protein in A375-R1 and A375-R3 cells compared with parental cells (supplemental Fig. S4). Together, these results confirm the activation of FGFR3 in the resistant cells possibly via an autocrine mode of activation.

**FGFR3 Activity Contributes to Resistance of A375-R1 Cells to Vemurafenib**

FGFR signaling has been previously implicated in resistance to EGFR inhibitors and Her-2-targeted therapies. These suggest an important role of the FGF/FGFR axis in the development of resistance to targeted therapies in cancer (30, 31). However, a role of FGFR signaling in resistance to B-RAF inhibition has not previously been reported. To verify our finding, we examined if FGFR3 activity is required for the resistance...
to vemurafenib. First, we investigated if FGFR3 is required for MAPK activation and maintaining resistance to vemurafenib in vemurafenib-resistant cells. We used LY2874455, an FGFR inhibitor described recently (32), and evaluated if targeting FGFR3 activity can re-sensitize A375-R1 cells to B-RAF inhibition. Interestingly, LY2874455 blocked the activation of MEK/ERK signaling in presence of vemurafenib as low as 100 nM (Fig. 4A). However, treatment with either LY2874455 or vemurafenib alone had no significant effect on the phospho-MEK or phospho-ERK levels in the A375-R1 cells (Fig. 4A). This indicates that combined inhibition of FGFR3 and B-RAF is required for total inhibition of MEK/ERK signaling in the vemurafenib-resistant cells. Consistent with our results with LY2874455 in A375-R1 cells, knockdown of FGFR3 by siRNA reduced MEK/ERK signaling in response to vemurafenib (Fig. 4B). Interestingly in A375-R1 cells, knockdown of FGFR3 by siRNA or LY2874455 treatment had minimal effect on pERK levels in the absence of vemurafenib. This suggests that in the absence of B-RAF inhibition, MEK/ERK signaling is driven primarily by mutant B-RAF in the A375-R1 cells (Fig. 4A). Together, we show that FGFR3 is required for sustained activation of MEK/ERK signaling in A375-R1 cells in the presence of vemurafenib inhibition.

Additionally, we tested if combined inhibition of B-RAF and FGFR3 activities could have anti-proliferation effect in vemurafenib-resistant cells. Inhibition of FGFR3 by FGFR inhibitor, LY2874455 or FGFR3-specific siRNA pool restored the sensitivity of A375-R1 cells to vemurafenib with IC_{50} values of 385 nM and 130 nM, respectively (Fig. 4, C and D). Together, our results suggest that FGFR3 is required for resistance to vemurafenib in A375-R1 cells and targeting FGFR3 could be an effective therapeutic strategy to overcome vemurafenib resistance.

FGFR3 Activation Induces Resistance to Vemurafenib in A375 Cells—To determine if FGFR3 activation can induce resistance to vemurafenib, we transfected the parental A375 cells with either empty vector or constitutively active FGFR3 K650E mutant expressing vector, and examined MEK/ERK signaling in response to B-RAF inhibition. We found that active FGFR3 K650E-expressing cells demonstrated up-regulated ERK activation in the presence of vemurafenib (Fig. 5A), but had no significant effect on the Akt activation. We also explored if bFGF stimulation is sufficient for inducing resistance to vemurafenib in the parental A375 cells. Interestingly, treatment of parental cells with bFGF induced a dose-dependent reactivation of MEK/ERK signaling in the presence of vemurafenib (Fig. 5B). FGFR3 was previously demonstrated to mediate its effects on MAPK pathway primarily via Ras activation (25). We thus analyzed if bFGF stimulation leads to Ras activation in A375 cells by immunoprecipitating Ras-GTP using GST-RBD beads. As demonstrated in Fig. 5C, stimulation of A375 cells with bFGF indeed induces Ras activation. Taken together, these results demonstrate that FGFR3 activity is sufficient for reactivation of Ras/Raf/MAPK signaling in B-RAF V600E melanoma cells by a mechanism that is independent of B-RAF kinase activity. Additionally, we evaluated the effect of FGFR3 activation on the sensitivity of A375 cells to vemurafenib. Expression of constitutively active FGFR3 or stimulation with bFGF attenuated the inhibitory effect of vemurafenib on the parental cells corre-
sponding to increased IC_{50} values of 1480 nM and 1190 nM, respectively (Fig. 5, D and E). However, bFGF failed to significantly affect the sensitivity of A375 cells to pharmacologic inhibitors of pan-RAF or MEK kinases suggesting that mechanism of bFGF induced resistance to B-RAF inhibition requires RAF/MEK activity (supplemental Fig. S5, A and B). Taken together, our results indicate that FGFR3 activation induces resistance to B-RAF inhibition in B-RAF V600E melanoma cells via reactivation of MAPK signaling.

**A375-R1 Cells Are Sensitive to MEK and pan-RAF Inhibition**—B-RAF V600E melanomas are addicted to the RAF/MEK/ERK signaling (6, 22). To investigate if vemurafenib-resistant cells rely on MAPK signaling for growth and proliferation, we evaluated the sensitivity of A375-R1 cells to pharmacologic inhibition of RAF or MEK kinases. We used AZD6244, a highly selective and potent inhibitor of MEK1/2, to test the effect of MEK inhibition on cell viability and ERK activity (33). AZD6244 inhibited cell proliferation in the parental and resistant cells with similar potency as indicated by IC_{50} values of 90 nM and 51 nM, respectively (Fig. 6A). In addition, AZD6244 strongly inhibited ERK activity in parental A375 as well as resistant A375-R1 cells (supplemental Fig. S6). Furthermore, we evaluated if RAF activities are required for sustained activation of MEK/ERK signaling in A375-R1 cells by using RAF265, a small molecule...
inhibitor of pan-RAF (34, 35). RAF265 inhibited the proliferation of the parental A375 and A375-R1 cells with similar potencies with IC_{50} values of 458 nM and 235 nM, respectively (Fig. 6B). Thus vemurafenib-resistant cells maintain their dependence on RAF/MEK/ERK signaling for cell proliferation. Previous reports have shown that alternate RAF isoforms can regulate the proliferation of vemurafenib-resistant melanomas via activation of MEK/ERK signaling (12, 17). Thus, we tested the RAF dependence of resistant cells by knockdown of all RAF isoforms via siRNA. Consistent with our results using pan-Raf inhibitor RAF265, treatment with siRNA targeting multiple RAF isoforms significantly reduced ERK activity and inhibited proliferation in A375 and A375-R1 cells (Fig. 6C and D). Taken together, our data suggest that the vemurafenib-resistant cells are still dependent on RAF/MEK/ERK activities and targeting this pathway remains a promising therapeutic strategy against B-RAF V600E melanomas, including ones resistant to B-RAF inhibition.

**DISCUSSION**

In this study, we generated three different resistant cell lines and showed that B-RAF V600E melanoma cells treated with B-RAF inhibitor developed resistance to B-RAF inhibition through Ras activation and subsequent reactivation of MAPK.
pathway. Additional molecular characterization revealed that enhanced FGFR3 signaling is involved in Ras activation and acquired-resistance to vemurafenib. These resistant cells maintain their addiction to the MAPK pathway as they are sensitive to the MEK or pan-RAF inhibition.

Consistent with the previous studies (12, 14, 17), we did not detect any additional B-RAF mutation in the vemurafenib-resistant cells, confirming that a secondary B-RAF mutation is not the mechanism of resistance to B-RAF inhibition. Although mutation in N-Ras or MEK have been reported in low frequency from B-RAF V600E melanoma patients who failed vemurafenib therapy (12, 16), we did not identify any mutation in Ras isoforms, the downstream RAF/MEK/ERK components or the upstream RTKs in the A375-R1 cells (data not shown), indicating that a non-genetic mechanism is involved in resistance to B-RAF inhibitors. In this study, we showed that active Ras-GTP levels are elevated in the vemurafenib-resistant B-RAF V600E melanoma cells (Fig. 2B). In addition, we showed that activation of FGFR3 induces Ras activation in B-RAF V600E melanomas and reduces their sensitivity to B-RAF inhibition (Fig. 5). Our findings indicate Ras activation as a critical node in mediating resistance to B-RAF-targeted therapies. Our data and others support this hypothesis as PDGFRβ (12, 18), IGF-1R (17) and N-Ras mutation (12) were identified as a B-RAF resistant mechanism in different cellular backgrounds. It is our speculation that Ras activation is a key mechanism involved in multiple resistance mechanisms to B-Raf inhibition.

FGFR signaling has been previously implicated in resistance to EGFR and Her-2 targeted therapies (30, 31). However, its role in resistance to B-RAF inhibition has never been reported. Here we describe FGFR3 signaling as an important player in the development of acquired resistance to vemurafenib. However,
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the resistant mechanism involving other RTKs particularly IGF-1R seems to require activation of alternate signaling pathways (i.e. PI3K/Akt) that may reduce the dependence of B-RAF V600E melanoma cells to RAF/MEK/ERK signaling (17). In this study however, we show that phospho-FGFR3 protein levels are up-regulated in the vemurafenib resistant B-RAF V600E melanoma cells (Fig. 3, A and B). Although we detected significant increase in the secretion of FGF2 ligand in the media by vemurafenib resistant B-RAF V600E melanoma cells, the precise mechanism of FGFR3 activation is currently under investigation (Fig. 3C and supplemental Fig. S4). Furthermore, we showed that FGFR3 signaling results in enhanced activation of downstream Ras/RAF/MEK/ERK signaling, thus conferring resistance to B-RAF inhibition (Figs. 3–5). In our resistant cells, no significant change in phospho-AKT status was observed when compared with the parental cells (Fig. 1D). In addition, activation of FGFR3 induced resistance to vemurafenib in B-RAF V600E cells without significantly affecting phospho-AKT levels (Fig. 5A), thus, MAPK reactivation is the dominant resistance mechanism in our resistant cells.

We also show that vemurafenib-resistant melanoma cells are equally sensitive to pan-RAF inhibitor RAF265 or MEK selective inhibitor AZD6244 when compared with parental cells, suggesting that vemurafenib-resistant cells indeed maintain their addiction to MAPK pathway (Fig. 6). Genetic depletion of Raf isoforms by siRNA in B-Raf resistant cells further confirmed these results (Fig. 6, C and D). Although, the precise role of individual RAF isoforms in resistance to B-RAF inhibition is yet to be fully investigated, our data are consistent with the earlier findings that B-RAF V600E melanoma cells can escape B-RAF kinase inhibition through MAPK reactivation by alternative RAF isoforms (12, 14, 15, 17). Therefore, a selective MEK inhibitor or a pan-Raf inhibitor may provide clinical benefit to melanoma patients who have failed or developed resistance to vemurafenib therapy.

Finally, we propose the following model to illustrate the mechanisms how B-RAF V600E melanoma cells develop resistance to vemurafenib treatment based on our results and other published studies (Fig. 7). When melanoma patients are treated with vemurafenib, two potential mechanisms of resistance can develop; a compensatory mechanism and/or genetic mutation. The compensatory mechanism we believe is the most common and dominant mechanism of resistance, and is mediated by one or more RTKs or other cell signaling component, such as COT (14). The genetic mutations identified and responsible for vemurafenib resistance include N-Ras Q61K/R mutation (12), K-Ras K117N (13), or MEK C1215 (16), and these mutations were confirmed in few patients who have relapsed from B-RAF inhibitor therapy. Thus, both compensatory mechanism and genetic mutations eventually lead to MAPK reactivation. Recently, dimerization of spliced form of BRAF V600E (p61) was also reported to induce MAPK pathway reactivation and resistance to vemurafenib (37). To date, activation of FGFR3, PDGFRβ, or IGF-1R was observed in different resistant cells, and the RTK(s) to be activated is likely context dependent. Importantly, activation of RTK leads to Ras activation, subsequent MAPK reactivation, and consequent drug resistance. Generally these resistant cells are still addicted to MAPK activity, and therefore, MAPK pathway inhibition by a pan RAF inhibitor or a MEK selective inhibitor could overcome their resistance to B-RAF inhibition. In certain context, in addition to MAPK reactivation, enhanced PI3K/AKT activities due to Ras activation or other cell signaling could contribute to the B-RAF resistance. Therefore, PI3K/AKT pathway inhibition could also be part of the strategy for overcoming resistance to B-RAF inhibitors.

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