Inhibiting EGF Receptor or SRC Family Kinase Signaling Overcomes BRAF Inhibitor Resistance in Melanoma

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ABSTRACT

We generated cell lines resistant to BRAF inhibitors and show that the EGF receptor (EGFR)-SRC family kinase (SFK)-STAT3 signaling pathway was upregulated in these cells. In addition to driving proliferation of resistant cells, this pathway also stimulated invasion and metastasis. EGFR inhibitors cooperated with BRAF inhibitors to block the growth of the resistant cells in vitro and in vivo, and monotherapy with the broad specificity tyrosine kinase inhibitor dasatinib blocked growth and metastasis in vivo. We analyzed tumors from patients with intrinsic or acquired resistance to vemurafenib and observed increased EGFR and SFK activity. Furthermore, dasatinib blocked the growth and metastasis of one of the resistant tumors in immunocompromised mice. Our data show that BRAF inhibitor-mediated activation of EGFR-SFK-STAT3 signaling can mediate resistance in patients with BRAF-mutant melanoma. We describe 2 treatments that seem to overcome this resistance and could deliver therapeutic efficacy in patients with drug-resistant BRAF-mutant melanoma.

SIGNIFICANCE: Therapies that target the driver oncogenes in cancer can achieve remarkable responses if patients are stratified for treatment. However, as with conventional therapies, patients often develop acquired resistance to targeted therapies, and a proportion of patients are intrinsically resistant and fail to respond despite the presence of an appropriate driver oncogene mutation. We found that the EGFR/SFK pathway mediated resistance to vemurafenib in BRAF-mutant melanoma and that BRAF and EGFR or SFK inhibition blocked proliferation and invasion of these resistant tumors, providing potentially effective therapeutic options for these patients. Cancer Discov; 3(2): 158-67. ©2012 AACR.
INTRODUCTION

The RAS–RAF–MEK (MAP–ERK kinase)–ERK (extracellular signal–regulated kinase) pathway is transiently activated downstream of receptor tyrosine kinases (RTK) and regulates a variety of cell responses to extracellular signals (1). However, in cancer, and in melanoma in particular, the pathway is constitutively activated and drives proliferation, survival, and tumor progression. A total of 3 RAS (HRAS, KRAS, and NRAS) and 3 RAF (ARAF, BRAF, and CRAF) genes are found in humans, and critically, NRAS, KRAS, and BRAF are mutated in 20%, 2%, and 45% of the melanomas, respectively (2).

Vemurafenib is a small-molecule drug that inhibits BRAF and achieves clinical responses in patients with BRAF-mutant melanoma, with improvements in progression-free and overall survival in BRAF-mutant, but not BRAF wild-type, melanomas (3, 4). Despite these impressive initial responses, most patients treated with vemurafenib develop resistance (acquired resistance) after a relatively short period of disease control. Furthermore, about 20% to 40% of patients do not respond to vemurafenib despite the presence of a BRAF mutation (intrinsic resistance). Thus, resistance is a persistent clinical problem in the management of BRAF-mutant melanoma, and second-line treatments are urgently required for patients with both intrinsic and acquired resistance to BRAF inhibitors.

Here, we show that EGF receptor (EGFR)–SRC family kinase (SFK)–signal transducers and activators of transcription 3 (STAT3) signaling is upregulated in BRAF inhibitor–resistant melanoma cells. We indicate that in addition...
to driving proliferation, this pathway drives invasion and metastasis. We also show that EGFR inhibitors cooperated with BRAF inhibitors to block the growth of resistant cells in vitro and in vivo. Further, we reveal that the broad-specifity tyrosine kinase inhibitor dasatinib inhibited growth and invasion of the resistant cells in vitro and in vivo. Moreover, we show that the EGFR was hyperphosphorylated in 4 of 5 tumors from patients with intrinsic or acquired resistance to vemurafenib. Finally, we show that SFK activation occurred in a resistant tumor and that dasatinib blocked the growth and metastasis of this tumor in immunocompromised mice. We conclude that EGFR–SFK–STAT3 signaling can mediate resistance to vemurafenib in patients with BRAF-mutant melanoma and describe 2 treatments that can overcome this resistance and could offer BRAF inhibitor–resistant patients vital second-line treatment options.

RESULTS

To investigate the molecular mechanisms of acquired resistance to BRAF inhibitors in melanoma, we cultured BRAF-mutant melanoma cells (A375, Colo829 cells) in increasing concentrations (up to 2 μmol/L) of the BRAF inhibitor PLX4720 (5). After approximately 2 months, we isolated resistant cell lines (A375/R; Colo829/R) that were 15- to 40-fold less sensitive to PLX4720 than the parental lines (Fig. 1A and B). In the second approach, we established A375 cells as tumor xenografts in nude mice and treated the mice with PLX4720 (25 mg/kg/d) until the tumors grew through the drug treatment. Cells derived from these tumors (A375(X)/R) were approximately 40-fold less sensitive to PLX4720 in vitro than cells derived from tumors from mice that had been treated with vehicle [A375(X); Fig. 1C]. Notably, although PLX4720 inhibited ERK activity in A375, A375(X), and Colo829 cells, it did not inhibit ERK activity in A375/R, A375(X)/R, or Colo829/R cells (Fig. 1D).

RTKs have been implicated in resistance to BRAF inhibitors (6–8), so we conducted phospho-array analysis to identify RTKs that were hyperactivated in the resistant lines, and consistent with previous studies (6–8), we observed increased phosphorylation of several RTKs (Fig. 1E). We were particularly intrigued to note that EGFR phosphorylation was increased in all 3 lines (Fig. 1E); although it is reported that this receptor is not expressed in melanoma cells (9), it can

Figure 1. The EGF receptor confers BRAF inhibitor resistance in BRAF-mutant melanoma cells. A–C, proliferation of A375 and A375/R cells (A), Colo829 and Colo829/R cells (B), and A375(X) and A375(X)/R cells (C) in the presence of PLX4720 (PLX). Values are relative to dimethyl sulfoxide (DMSO) controls and IC50 values are mean (μmol/L; n = 3) ± SEM (34). D, Western blot analyses for phosphorylated ERK (pERK) and ERK2 (loading control) in A375, A375/R, A375(X), A375(X)/R, Colo829, and Colo829/R cells in the absence (−) or presence (+) of PLX4720 (2 μmol/L; 24-hour continuous exposure). E, phospho-protein array for A375, A375/R, A375(X), A375(X)/R, Colo829, and Colo829/R cells. VEGFR, VEGF receptor. (continued on following page)
mediate intrinsic resistance of colorectal cancer cells to vemurafenib (10, 11). EGFR has several tyrosine phosphorylation sites, so to determine functionality, we used Western blotting to confirm that phosphorylation of the activation site Y1068 was elevated in the resistant lines (Fig. 1F). We also show that phosphorylation of AKT, a downstream signaling target, was increased (Fig. 1F).

Recent studies have shown that growth factors can mediate resistance to vemurafenib (12, 13), and we show that EGFR secretion was increased in all 3 resistant lines (Fig. 1G). Note that EGFR expression was also increased (Fig. 1F). We used quantitative mass spectrometry by stable isotope labeling of amino acids in cell culture (SILAC) to examine the phosphoproteome in resistant cells. We identified approximately 4,500 unique phospho-peptides, of which approximately 800 were differentially phosphorylated in sensitive and resistant cells (Supplementary Table S1). Notably, 3 of the differentially phosphorylated phospho-peptides were from MIG6, a negative regulator of the EGFR (14–18). Critically, the sites that are implicated in negative regulation of EGFR signaling (S249, S251; ref. 19) were dephosphorylated in the resistant cells (Supplementary Table S2), and we confirmed by Western blot analysis that MIG6 phosphorylation was reduced in the resistant cells (Fig. 1H). Notably, as with colorectal cells (10, 11), despite EGFR activation, the resistant cells were insensitive to gefitinib (an EGFR inhibitor) monotherapy but were sensitive to the gefitinib and PLX4720 combination in vivo (Fig. 1I) and in vitro (Fig. 1J).

To investigate which pathways downstream of EGFR modulated BRAF inhibitor resistance, we used phosphoprotein arrays to compare the activity of 46 cancer-related cytosolic kinases and selected transcription factors in A375 and A375/R cells. In accordance with previous studies linking SRC to BRAF inhibitor resistance in cell lines (20), we observed elevated phosphorylation of the SFKs LYN, YES, and FYN (Fig. 2A and B) in the resistant lines. We confirmed by Western blotting that LYN phosphorylation was increased in the A375(X)/R, A375/R, and Colo829/R cells (Fig. 2C).
Figure 2. The SFKs confer BRAF inhibitor resistance in BRAF-mutant melanoma cells. A, phospho-protein array from A375 and A375/R cells. B, quantitation of phospho-array data for LYN, FYN, YES, and FAK from A. C, Western blot analysis showing LYN phosphorylation (pLYN) in A375(X), A375(X)/R, A375, A375/R, Colo829, and Colo829/R cells. Tubulin, loading control. D, proliferation of A375, A375/R, A375(X), A375(X)/R, Colo829, and Colo829/R cells in the presence of dasatinib and saracatinib. Values are relative to DMSO, and IC\textsubscript{50} values are presented as mean (μmol/L; n = 3) ± SEM. E, graph showing growth of A375(X)/R xenografts in nude mice treated with vehicle, PLX4720 (25 mg/kg/d orally), or dasatinib (75 mg/kg/d orally). Drug treatments commenced when tumors were 40 to 50 mm\textsuperscript{3} and mean tumor volumes ± SEM are shown (n = 6 mice/group). F, Western blot analyses for phospho-SFK Y416 (pSFK) and SRC in A375(X), A375(X)/R, and Colo829/R cells treated with DMSO or dasatinib (DAS; 20 nmol/L, 24 hours). G, photomicrographs showing pSFK staining in A375(X)/R xenografts from mice after 30 days of treatment with vehicle or dasatinib (75 mg/kg/d orally). Scale bar, 50 μm.
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We show that compared with their respective parental lines, A375(X)/R, A375/R, and Colo829/R cells were more sensitive to the pan-SFK inhibitors dasatinib and saracatinib in vitro (Fig. 2D) and that dasatinib, but not PLX4720, suppressed A375/R tumor xenograft growth in nude mice (Fig. 2E). Accordingly, using a phospho-specific antibody that binds to several of the SFKs, we show that dasatinib suppressed SFK phosphorylation in the resistant cells in vitro (Fig. 2F), but without affecting EGFR phosphorylation (Supplementary Fig. S1), and it induced loss of phosphorylated SFKs in the membranes of A375/R tumor xenografts (Fig. 2G and Supplementary Table S3).

While conducting this work, in a parallel study, we discovered that BRAF inhibitors increase invasion of drug-resistant melanoma cells (B. Sanchez-Laorden and colleagues; submitted for publication). We confirmed that the 3 drug-resistant cell lines used here were more invasive in vitro than their respective parental cells (Fig. 3A). We performed Gene Ontology (GO) modular enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (refs. 21, 22; Supplementary Table S4) to analyze our SILAC phospho-proteome. We found that the highest enrichment group comprised 91 cytoskeleton proteins (enrichment score 33.36 at medium stringency) that form a functional protein–protein interaction network involved in invasion (Fig. 3B), when analyzed by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; ref. 23). To examine the in vivo consequences of these observations, we injected A375(X) and A375(X)/R cells into the tail veins of nude mice and show that the A375(X)/R cells seeded the lungs more efficiently than did the A375(X) cells (Fig. 3C). Critically, we indicate that invasion was blocked when the cells were treated with gefitinib or dasatinib (Fig. 3D) or transfected with siRNA selective for SRC or LYN (Fig. 3E).

In addition to revealing increased SFK phosphorylation, the reverse arrays showed increased phosphorylation of several STAT family members (Figs. 2A, 3F). We confirmed that STAT3 phosphorylation was elevated in the resistant cells (Fig. 3G). Importantly, STAT proteins are activated downstream of cytokine and growth factor receptors, including EGFR (24), and their constitutive activation is implicated in invasion and metastasis in various cancers (25). Accordingly, we show that STAT3 phosphorylation was suppressed by gefitinib and dasatinib, but not by a pan-Janus kinase (JAK) inhibitor (Fig. 3H). We also show that the STAT3 dimerization inhibitor, S31-201, blocked STAT3 phosphorylation (Fig. 3H) and decreased invasion of the resistant lines (Fig. 3I), but without affecting their viability (data not shown).

The data above show that the EGFR–SFK–STAT3 pathway can drive proliferation, invasion, and lung colonization in drug-resistant BRAF-mutant melanoma cells. To evaluate the clinical relevance of these findings, we examined EGFR signaling in melanoma patients with intrinsic or acquired resistance to vemurafenib. We show that phospho-EGFR staining was increased in 4 of 5 resistant tumors (Fig. 4A and Supplementary Fig. S2). Notably, we obtained paired samples from one of these patients, and these show a clear increase in phospho-EGFR (Fig. 4A) and phospho-SFK staining in the posttreatment compared with the pretreatment tumor (Fig. 4B). We propagated the posttreatment tumor from this patient in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, and as reported in other tumor types (26), these xenotumors presented the same pathologic and histologic features as the patient's original tumor and metastasized to the lymph nodes, lungs, and liver (data not shown), confirming that it adopted the same growth patterns in the mice as in the patient.

A critical observation is that orally administered dasatinib (75 mg/kg/d) reduced SFK phosphorylation (Fig. 4B and C, Supplementary Table S5) and suppressed the growth of xenotumors in the mice (Fig. 4D; P < 0.035). Furthermore, although 4 of 9 (44.4%) animals from the vehicle-treated cohort presented large metastatic HMB45 deposits in at least one regional lymph node basin (Fig. 4E), none of the dasatinib-treated animals showed lymphatic involvement (data not shown). We also observed that 8 of 9 mice in the vehicle-treated cohort presented metastatic deposits in the lungs that were composed of cells with morphology similar to that of the original tumor and stained positive for HMB45 (Fig. 4G). In general, tumors in the vehicle-treated animals were large enough to be seen by hematoxylin and eosin (H&E), and in 2 animals were particularly large and well established (Fig. 4F and Supplementary Table S6). In contrast, no tumors visible by H&E were observed in the dasatinib-treated animals, although rare HMB45-positive cells could be observed microscopically (Fig. 4F and Supplementary Table S6).

**DISCUSSION**

Although BRAF inhibitors have shown great promise for the management of patients with melanoma, both intrinsic and acquired drug resistance are persistent clinical problems, so new treatments are urgently needed for resistant patients and are likely to materialize from improved knowledge of the molecular mechanisms underlying resistance. By generating and characterizing drug-resistant cell lines in vitro and in vivo, we have shown that EGFR–SFK–STAT3 signaling can mediate resistance to BRAF inhibitors in melanoma. It was previously reported that melanoma cells do not express EGFR (11). However, this finding was as judged relative to colorectal carcinoma and thyroid cancer cells, which express very high levels of this receptor. We clearly establish that EGFR is expressed in melanoma cells and that it can mediate their resistance to vemurafenib. We observed increased EGFR phosphorylation in the resistant cells, and this was accompanied by increased expression of EGFR itself, increased secretion of EGF, and decreased phosphorylation of MIG6. We posit that EGFR activity is increased by the coordinated action of several pathway components, a conclusion supported by our observation that MIG6 depletion by RNA interference modulated only a modest (4-fold) increase in resistance of melanoma cells to BRAF inhibitors (data not shown).

We show that in addition to stimulating the growth of the resistant cells, this pathway also stimulates invasion and metastasis. These results are consistent with recent data from our laboratory indicating that BRAF inhibitors induce invasion through paradoxical activation of the ERK pathway in melanoma cells (B. Sanchez-Laorden and colleagues; submitted for publication) and a study showing that SRC and MEK inhibitors cooperate to block invasion in drug-sensitive
Figure 3. BRAF inhibitor-resistant BRAF-mutant melanoma cells show increased invasion and metastatic potential. A, in vitro invasion of A375, A375/R, A375(X), A375(X)/R, Colo829, and Colo829/R cells into collagen I matrices. Data are representative of 3 independent experiments, with error bars to indicate SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, protein interaction network showing 91 differentially expressed phosphoproteins from the cytoskeleton cluster in A375 and A375/R cells. The network was generated using STRING (23). Color coding is used to show evidence for the types of interactions between the individual phospho-proteins. C, quantification of the tumor area in the lungs of mice following tail vein injection with A375(X) or A375(X)/R cells. D, invasion of A375/R, A375(X)/R, and Colo829/R cells in the presence of DMSO, gefitinib (2 μmol/L), and dasatinib (10 nmol/L). Data are representative of 3 replicates from 3 independent experiments, with error bars to indicate SE. *, P < 0.05; **, P < 0.01. E, invasion of A375/R, A375(X)/R, and Colo829/R cells transfected with scrambled control (SC), SRC (siSRC), or LYN (siLYN) siRNA. Data are representative of 3 replicates from 3 independent experiments, with error bars to indicate SE. *, P < 0.05; **, P < 0.01. Western blots show SRC and LYN expression. Tubulin: loading control. F, quantitation of phospho-array data for STAT family members in Fig. 2A. G, Western blot analyses for phosphorylated STAT3 (pSTAT3) and STAT1 (loading control) in A375, A375/R, A375(X), A375(X)/R, Colo829, and Colo829/R cells. H, Western blot analyses for phosphorylated STAT3 (pSTAT3) and STAT3 (loading control) in A375(X)/R cells treated with DMSO, gefitinib (GEF, 2 μmol/L), dasatinib (DAS, 10 nmol/L), the JAK3 inhibitor P6 (JAK3i, 2 μmol/L), PLX4720 (PLX, 2 μmol/L), and S3I-201 (20 μmol/L). I, A375/R cell invasion in the presence of DMSO or S3I-201 (50 μmol/L). Data are representative of 3 independent experiments, with error bars to indicate SE. *, P < 0.05; **, P < 0.01.
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**Figure 4.** Dasatinib overcomes the growth and metastatic spread of vemurafenib-resistant tumors. **A,** photomicrographs showing pEGFR staining in pretreatment and post-vemurafenib-treated tumor samples from a patient who displayed intrinsic resistance to vemurafenib. Scale bar, 100 μm. **B,** photomicrographs showing H&E and phospho-SFK (pSFK) staining in pretreatment and post-vemurafenib-treated tumors from the patient samples shown in **A.** Scale bar, 50 μm. **C,** photomicrograph showing phospho-SFK (pSFK) staining in xenotumors of vemurafenib-resistant tumors grown in NOD/SCID mice and treated with vehicle or dasatinib (75 mg/kg/d orally). Scale bar, 250 μm. **D,** graph showing phospho-SFK (pSFK) levels in lysates from xenotumors of vemurafenib-resistant tumors grown in NOD/SCID mice and treated with vehicle or dasatinib (75 mg/kg/d orally). The graph shows quantification by densitometry of signals from Western blots (shown below; n = 6 for dasatinib group; n = 8 for vehicle group) using ImageJ Software to quantify the Western blot data. **E,** growth of vemurafenib-resistant xenotumors in NOD/SCID mice treated with vehicle or dasatinib (75 mg/kg/d orally). Drug treatments commenced when tumors were approximately 200 mm³ and show mean tumor volumes ± SEM (ref. 34; n = 6 for dasatinib group; n = 9 for vehicle group). **F,** photomicrographs showing H&E and HMB45 staining of a lymph node from vehicle-treated NOD/SCID mouse bearing vemurafenib-resistant tumor xenotumors. Scale bar, 75 mm. **G,** photomicrographs showing H&E and HMB45 staining of lungs from NOD/SCID mice bearing vemurafenib-resistant tumor xenotumors and treated with vehicle or dasatinib (75 mg/kg/d orally). Scale bar, 75 mm. P, parenchyma; T, tumor.
melanoma cells (27). The growth of resistant cells was blocked by a combination of EGFR and BRAF inhibitors in vitro and in vivo. Furthermore, the broad-specificity tyrosine kinase inhibitor dasatinib inhibited growth and invasion of the resistant cells in vitro and blocked their growth and metastasis in vivo. Dasatinib inhibits nearly 40 different protein kinases, including the SFKs and other non-RTKs such as FRK, BRK, and ACK (28). However, we posit that the SFKs play a key role in mediating resistance to BRAF inhibitors in melanoma cells. We show that the growth of the resistant cells was sensitive to dasatinib and saracatinib, 2 distinct pharmacophore inhibitors of these kinases. We found that dasatinib and depletion of SRC and LYN both suppressed invasion of the resistant cells in vitro. Of critical importance, SFK signaling was increased in a tumor from a patient with intrinsic resistance to vemurafenib, and we reveal that dasatinib inhibited the growth and metastasis of this tumor in mice.

Recent data have established that melanoma resistance to BRAF inhibitors can be mediated by several mechanisms owing to tumor heterogeneity and plasticity (6–8). Our results highlight the potential of propagating patient-derived tumor tissues in immunocompromised mice as an approach to evaluating possible responses to targeted therapies. We posit that the combination of EGFR plus BRAF inhibitors may prevent the emergence of at least some forms of resistance in patients, and that broad specificity tyrosine kinase inhibitors—such as dasatinib—that target both RTKs and SFKs could overcome some forms of intrinsic and acquired resistance. These 2 therapeutic approaches could therefore provide efficacy in patients for whom no effective treatments are currently available.

METHODS

See online supplementary data for details. A375 and Colo829 cells were obtained from the American Type Culture Collection and were authenticated in house by short tandem repeat profiling before commencing the study and again while the experiments were being carried out. Cells were cultured in Dulbecco modified Eagle medium (DMEM) or RPMI-1640 supplemented with 10% FBS. In vitro invasion assays were as described (29). SMARTpool siRNA duplexes were transfected using Lipofectamine 2000 (Invitrogen), and cell viability was assessed by CellTiter-Glo (Roche). Antibody arrays were conducted using ARY001 and ARY003 kits (R&D Systems) and analyzed by ImageJ (32). Tumor samples from patients were collected into DMEM supplemented with FBS (2%), gentamicin (50 μg/mL), and Fungizone (12.5 μg/mL) and maintained subcutaneously in the flanks of NOD/SCID mice. Phospho-proteomics was conducted by SILAC (33).

Disclosure of Potential Conflicts of Interest

D. Niculescu-Duvaz, A. Zambon, and C.J. Springer are employees of the Institute of Cancer Research, which operates a Rewards for Inventors scheme. They have been developing RAF inhibitors, but these inhibitors are not mentioned in this article. P. Lorigan receives honoraria from the speakers’ bureau of Roche Pharmaceuticals. J. Larkin is a consultant/advisory board member of GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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