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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

EGFR-Mediated Reactivation of MAPK Signaling Induces Acquired Resistance to GSK2118436 in BRAF V600E–Mutant NSCLC Cell Lines

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Abstract

Although treatment of BRAF V600E–mutant non–small cell lung cancer (NSCLC^{V600E}) with GSK2118436 has shown an encouraging efficacy, most patients develop resistance. To investigate the mechanisms of acquired resistance to GSK2118436 in NSCLC^{V600E}, we established GSK2118436-resistant (GSR) cells by exposing MV522 NSCLC^{V600E} to increasing GSK2118436 concentrations. GSR cells displayed activated EGFR–RAS–CRAF signaling with upregulated EGFR ligands and sustained activation of ERK1/2, but not MEK1/2, in the presence of GSK2118436. Treatment of GSR cells with GSK2118436 enhanced EGFR-mediated RAS activity, leading to the formation of BRAF–CRAF dimers and transactivation of CRAF. Interestingly, sustained activation of ERK1/2 was partly dependent on receptor-interacting protein kinase-2 (RIP2) activity, but not on MEK1/2 activity. Combined BRAF and EGFR inhibition blocked reactivation of ERK signaling and improved efficacy *in vitro* and *in vivo*. Our findings support the evaluation of combined BRAF and EGFR inhibition in NSCLC^{V600E} with acquired resistance to BRAF inhibitors. *Mol Cancer Ther; 15(7); 1627–36. ©2016 AACR.*

Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide (1). Over the past decade, molecularly targeted therapies, which block important oncogenic pathways, have made remarkable progress. Treatment with EGFR and anaplastic lymphoma kinase tyrosine kinase inhibitors (TKI) in molecularly selected population has led to unprecedented improvements in objective response rate (ORR) and progression-free survival over cytotoxic chemotherapy (2–4).

Aberrant activation of the MAPK pathway, which plays a major role in cell proliferation, survival, and differentiation, contributes to various types of cancers. BRAF is a serine/threonine kinase of the RAF family kinases, which also include ARAF and CRAF. BRAF serves as a central intermediate in transmitting extracellular signals to the dual-specificity protein kinase MEK1/2, which in turn activates ERK1/2 (5). BRAF is commonly activated by a somatic mutation at codon 600 (V600E) in various tumor types, including 40% to 50% of cutaneous melanomas and 10% of colorectal cancers (6). The RAF–MEK–ERK pathway is a key downstream effector of the small GTPase RAS, the most frequently mutated oncogene in human cancers (7). Furthermore, RAS is a key downstream effector of the growth factor receptor, which is mutationally activated and/or overexpressed in multiple tumor types (5, 7).

Oncogenic BRAF V600E mutations are present in approximately 1.3% of NSCLCs and are thought to result in constitutive kinase activation and downstream phosphorylation (8). The finding that lung-specific expression of the BRAF V600E mutation in mouse models leads to development of invasive adenocarcinoma further supports the potential role of BRAF V600E as an oncogenic mutation in NSCLC (9). Very recently, a phase II trial of a BRAF inhibitor, GSK2118436 (dabrafenib), showed significant antitumor activity with a reported ORR of 32% in advanced BRAF V600E–mutant NSCLC (NSCLC^{V600E}; NCT01336634). However, clinical efficacy of the BRAF inhibitor is limited by the emergence of drug resistance, as responses are transient and tumors eventually recur. These clinical observations make it crucial to understand the mechanisms underlying resistance to BRAF inhibitors and to identify therapeutic strategies for treatment of NSCLC^{V600E} with acquired resistance to BRAF inhibitors.

In this study, our objective was to understand the acquired resistance mechanism to BRAF inhibitors in NSCLC^{V600E}.

Materials and Methods

Cell lines

MV522 were obtained from Dr. Alex A. Adjei (Roswell Park Cancer Institute, Buffalo, NY) in March 2014. HCC364 human lung cancer cell line harboring the BRAF^{V600E} mutation was

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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obtained from Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX) and A375 human melanoma cell lines were obtained from Korea Cell Line Bank in March 2016. Cell lines were maintained at 37°C in a humidified atmosphere at 5% CO_2 and grown in RPMI1640 media supplemented with 10% FBS, 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (HyClone, GE Healthcare). Authetication of the cell lines was done using short tandem repeat analyses.

Chemicals, reagents, and antibodies

GSK2118436 and gefitinib were purchased from Selleckchem. Cetuximab was supplied by ImClone Systems. Anti-phospho-EGFR (Y1068; #3777), anti-EGFR (#2232), anti-phospho-BRAF (S445, #2696), anti-BRAF (#9433), anti-phospho-CRAF (S338, #9427), anti-CRAF (#9422), anti-phospho-ERK (T202/Y204, #9101), anti-ERK (#9106), anti-phospho-AKT (S473, #9271), anti-AKT (#9272), anti-phospho-MEK (S217/221, #9154), anti-MEK (#9126), anti-phospho-RIP2 (S176, #14397), RIP2 (#4142), anti-phospho-MET (Y1234/1235, #3129), and anti-MET (#8198) were purchased from Cell Signaling Technology. Anti-β-actin (#A3854) was purchased from Sigma-Aldrich. All donkey anti-rabbit IgG-HRP (#sc-2077) and donkey anti-mouse IgG-HRP (#sc-2096) were purchased from Santa Cruz Biotechnology.

Cell viability

MV522 cells were cultured in increasing concentrations of GSK2118436 from 0.01 to 3 μmol/L to generate polyclonal resistant clones (GSR pool). Single-cell clones (GSR#1 and GSR#2) were isolated by limiting dilution from GSR pool. For MTT assay, cells were seeded in 96-well plates (2×10^3 cells/well) for 24 hours. Inhibitors were added and incubated for 72 hours. After MTT solution was added, plates were incubated for 4 hours, and the results were measured at 540 nm. For colony formation assay, cells were plated into 6-well plates (5×10^4 cells/well) and treated with inhibitors. After 14 days, cells were fixed in 3% paraformaldehyde and stained with 0.005% crystal violet.

gDNA extraction and Sanger sequencing

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen; #69504) according to the instructions of the manufacturer. *BRAF* and *RAS* genes were amplified from genomic DNA by PCR. PCR products were purified using QIAquick PCR Purification Kit (Qiagen; #28104), followed by Sanger sequencing using previously reported primer (listed in Supplementary Table S1) set (1).

siRNA knockdown

siRNA was synthesized by IDT (the sequence of siRNA listed in Supplementary Table S2) and was transfected into the cells using Lipofectamine RNAiMAX (Invitrogen; #13778) according to the manufacturer's instructions.

RAS activity assay

Ras activity was measured using the Ras Activation ELISA Assay Kit (Cell Biolabs; #STA441). Briefly, cell lysates were incubated with Raf1-Ras–binding domain (RBD) agarose. Raf1-RBD was used to capture the active GTP-bound Ras protein, which was then detected by the addition of an anti-Ras-antibody. An HRP-conjugated secondary antibody was then added. A luminometer was used to measure the signals after addition of a chemiluminescent reagent (PerkinElmer).

Receptor tyrosine kinase arrays

The Human Phospho-RTK Array Kit (R&D Systems; #ARY001B) was used to detect kinase activation within a panel of receptor tyrosine kinases (RTK) according to the manufacturer's instructions. Briefly, MV522 cells and GSR (pool) cells were plated in 10-cm dishes and collected after 24 hours. Lysate was applied to a membrane-anchored RTK array and incubated at 4°C for 24 hours. Membranes were exposed to chemiluminescent reagents and detected by X-ray film (AGFA).

Immunoblotting

Cells were washed in PBS and lysed in a lysis buffer (Cell Signaling Technology; #9803). Protein concentrations were determined by the Bradford Assay Kit (Bio-Rad). Equal amounts of cell extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes (Millipore), and immunoblotted with specific primary and secondary antibodies. Membranes were exposed to chemiluminescent reagents and detected by X-ray film (AGFA).

IHC

Sacrificed tumors were fixed in 10% neutral-buffered formalin and paraffin embedded. Endogenous peroxidase activity was quenched with 10% hydrogen peroxide in methanol. The sections were blocked with normal serum for 1 hour and then incubated overnight with anti-p-EGFR (Y1068), anti-phospho-ERK, Ki67 (Santa Cruz Biotechnology; #sc-23900), and anti-pRIP2 (S176; 1:200). The peroxidase-conjugated secondary antibodies (Dako) were incubated for 1 hour at room temperature. Staining intensity was scored as 0 (absent), 1+ (low-moderate), and 2+ (high).

RT-PCR and real-time PCR assay

RNA was extracted using the RNeasy mini Kit (Qiagen; #74104) according to the instructions of the manufacturer. Extracted RNA was used for the synthesis of cDNA by SuperScript III First-Strand Synthesis SuperMix (Invitrogen), following the kit manual, and RT-PCR was performed on Applied Biosystems StepOnePlus with SYBR-Green PCR Master Mix (Life Technologies). GAPDH expression was used as an internal reference to normalize input cDNA.

Xenograft studies

Animal care was performed in accordance with institutional guidelines. Treatment was by oral gavage daily with vehicle, 300 mg/kg GSK2118436, gefitinib 50 mg/kg, or the last two together. Tumor size was determined by caliper measurements of tumor length, width, and depth, and volume was calculated as volume = 0.5236 × length × width × depth (mm).

Statistical analysis

Quantitative results were analyzed by one-way ANOVA or *t* test. Statistical significance was established for $P < 0.05$.

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Table 1. Characterization of MV522 cells and resistant clones to GSK2118436

Cells	NRAS	KRAS	HRAS	BRAF	BRAF exon 1–14, 16–18	MEK1	IC_{50} (μmol/L)
MV522	N/D	N/D	N/D	V600E	N/D	N/D	0.00176
GSR (Pool)	N/D	N/D	N/D	V600E	N/D	N/D	>10
GSR#1	N/D	N/D	N/D	V600E	N/D	N/D	>10
GSR#2	N/D	N/D	N/D	V600E	N/D	N/D	>10

NOTE: Mutation status of *BRAF*, *MEK*, and *RAS* genes are summarized for MV522 cells and GSR cells.
Abbreviation: N/D, not detected.

Results

Establishment and characterization of BRAF V600E–mutant NSCLC cells with acquired resistance to GSK2118436

To explore the mechanisms of acquired resistance to GSK2118436 in lung cancer, BRAF V600E–mutant MV522 lung adenocarcinoma cells were cultured in increasing concentrations (up to 3 μmol/L) of the inhibitor. After approximately 3 months, we established cells (GSR-pool, GSR#1, GSR#2) with strong resistance to GSK2118436 (IC_{50} > 10 μmol/L; Fig. 1A; Table 1). Using an MTT assay, we observed the dose-dependent antiproliferative effect of GSK2118436 in MV522 cells, but growth inhibition was not detected in GSR (pool), GSR#1, and GSR#2 (Fig. 1B). These results correlated with results obtained by a long-term (2 weeks) colony formation assay (Fig. 1C).

Like in BRAF V600E–mutant melanoma (melanoma^{V600E}), bidirectional Sanger sequencing of all 18 *BRAF* exons in all three GSR clones revealed no gatekeeper mutations in *BRAF* and retention of the original *BRAF* V600E mutation (Fig. 1D; Table 1; ref. 10). Furthermore, there was no secondary *NRAS*, *KRAS*, *HRAS*, or *MEK1* mutation, which could explain resistance to GSK2118436 (Table 1).

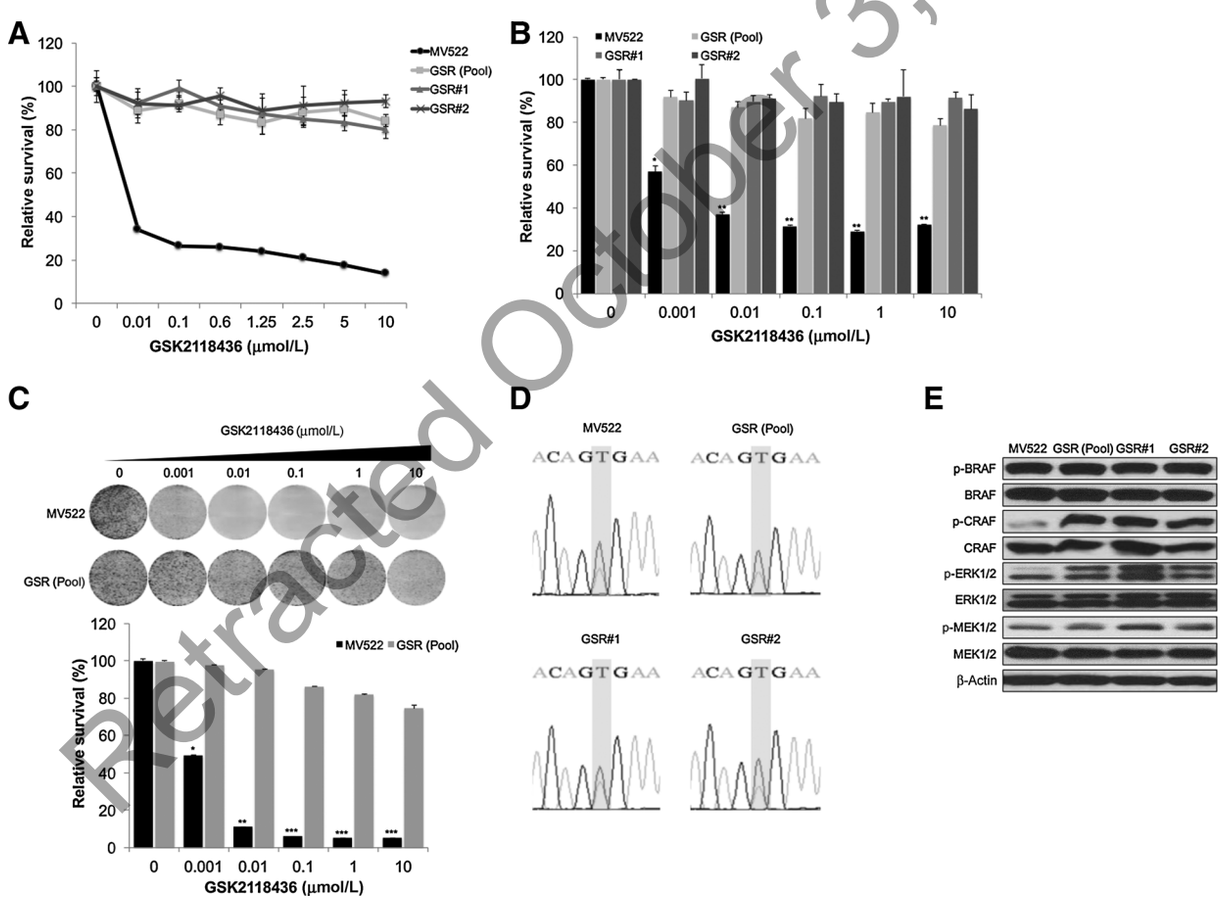

Figure 1.
Establishment and characterization of GSK2118436-resistant clones. A, relative survival of MV522 cells and GSK2118436-resistant clones (GSR-pool, GSR#1, GSR#2) after treatment of GSK21128436 for 72 hours. B, in MTT assays, all cells were treated with the indicated concentrations of GSK2118436 for 72 hours. C, in colony formation assays, cells were grown in the absence or presence of the indicated concentrations of GSK2118436 for 14 days. All cells were fixed, stained with crystal violet (0.005%), and photographed. Photographs of fixed cells are positioned with graphs to represent quantification. All data are displayed as mean ± SEM ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). D, Sanger sequencing of DNA from each cells showing retention of BRAF (V600E) mutation. E, Western blot analysis of all cell lysates with indicated antibodies.

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Compared with MV522 cells, GSR cells exhibited higher basal levels of p-ERK1/2, suggesting that the mechanism of acquired resistance may be ERK-dependent (Fig. 1E). ERK1/2 activation in GSR cells was associated with CRAF phosphorylation at S338, indicative of activation of CRAF kinase (Fig. 1E). Interestingly, the robust increase of p-ERK1/2 levels in GSR cells were seen without clear evidence of MEK1/2 activation. Together, these data suggest that high basal levels of p-CRAF and p-ERK1/2, but not acquired mutations in MAPK pathway genes, might account for the resistance to GSK2118436 in GSR cells.

GSK2118436-resistant clones display sustained phosphorylation of ERK that is independent of MEK1/2

We further examined whether resistant clones are dependent on sustained activation of CRAF and ERK1/2 for survival (Fig. 2A and B). Consistent with the cell viability data, Western blot analysis showed that GSK2118436 treatment significantly suppressed both p-MEK1/2 and p-ERK1/2 in MV522 cells, whereas it suppressed p-MEK1/2, but not p-ERK1/2, in GSR clones (Fig. 2A). In melanoma^{V600E}, ERK1/2 inactivation following BRAF inhibition was reported to relieve the negative feedback suppression of upstream RTK/RAS signaling, leading to the dimerization and activation of RAF isoforms (11). Consistent with previous findings in melanoma^{V600E}, acute ERK1/2 inactivation following GSK2118436 treatment induced CRAF activation in MV522 cells (Fig. 2A). However, CRAF and ERK1/2 are constitutively activated regardless of GSK2118436 treatment in GSR clones. The constitutive activation of CRAF in GSR clones suggests that there may be constitutive upstream activation, such as an *NRAS* mutation (which was excluded by Sanger sequencing; Supplementary Table S1) or RTK activation. Strikingly, unlike melanoma and colorectal cancer (10, 12–15), GSK2118436 treatment resulted in dose-dependent suppression of MEK1/2 activity but resulted in no significant alterations in p-ERK1/2 levels in GSR clones (Fig. 2A). The pharmacologic uncoupling between MEK1/2 and ERK1/2 activity suggests that MEK1/2 may not directly regulate ERK1/2 activity in GSR clones.

The differential dependence of MV522 and GSR clones on MEK1/2 signaling for cell survival was clearly shown in cell viability assays (Fig. 2B). AZD6244, a MEK1/2 inhibitor, potently inhibited cell growth of MV522 cells in a dose-dependent manner. In contrast, GSR clones exhibited resistance at high doses of AZD6244. Combined inhibition of BRAF and MEK is associated with complete inhibition of the MAPK pathway in melanoma^{V600E}, leading to enhanced cytotoxicity and clinical activity (16). In GSR cells, however, cotreatment of GSK2118436 and AZD6244 did not show synergistic growth inhibition, which further supports the existence of signaling pathway for ERK1/2 activation bypassing MEK1/2 inhibition (Fig. 2C).

Together, these data suggest that activated CRAF may transduce prosurvival signals to ERK1/2 and that sustained activation of ERK1/2 is independent of MEK1/2 activity.

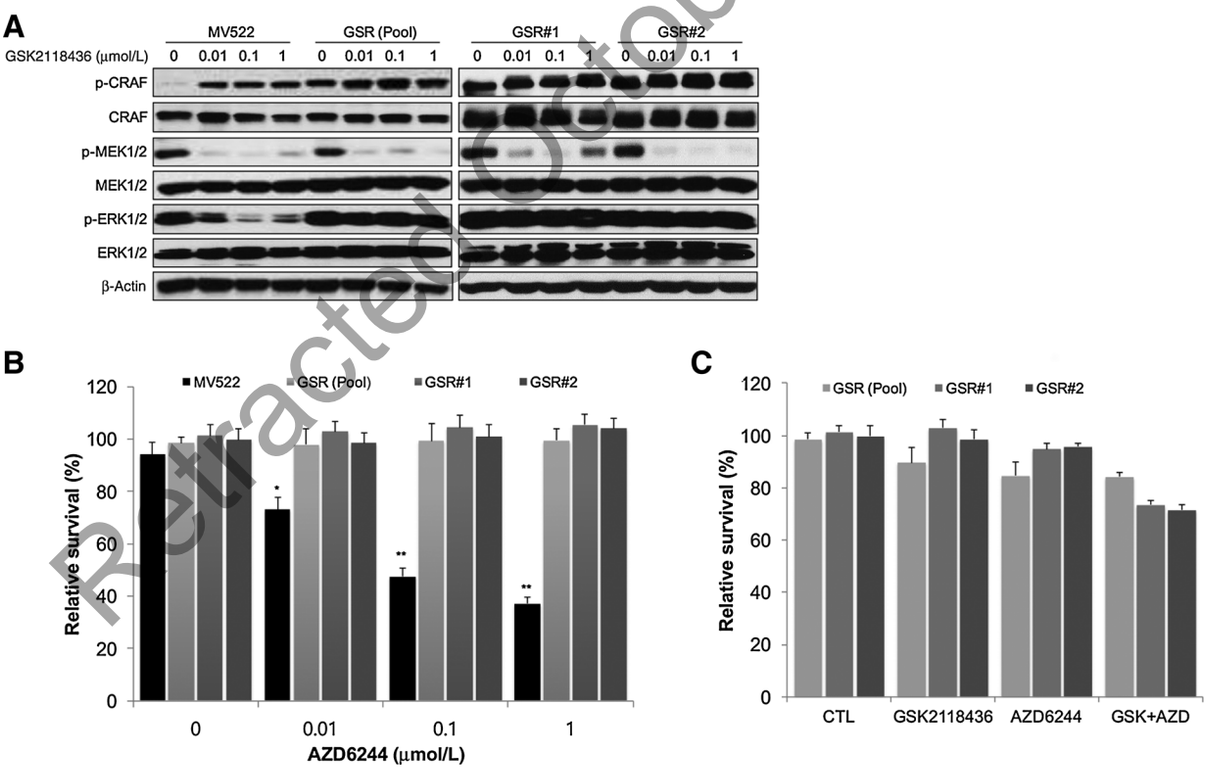

Figure 2.

ERK1/2 activation is independent of MEK1/2 activity in GSR clones. A, cells were treated with GSK2118436 at the indicated concentration, and lysates were probed with the indicated antibodies. B, in MTT assays, cells were treated with the indicated concentration of AZD6244 for 72 hours (*, $P < 0.05$; **, $P < 0.01$). C, MV522 cells and GSR cells were treated in control (CTL) or with 0.1 μmol/L GSK2118436 or 1 μmol/L AZD6244 (AZD) or in their combination for 72 hours. All data are displayed as mean ± SEM ($n = 3$).

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Autocrine HB-EGF–EGFR signaling confers acquired resistance to GSK2118436 via activation of RAS-CRAF

In melanoma^{V600E}, RTK activation increases the level of RAS-GTP, promotes dimerization of RAF isoforms, and reactivates ERK signaling, leading to resistance to BRAF inhibitors (12). Given the constitutive CRAF activation in GSR clones, we hypothesized that phosphorylation of CRAF may be mediated by activated RTKs. To determine whether activation of RTK signaling might account for the observed CRAF activation, we performed phospho-RTK arrays in MV522 and GSR cells. Interestingly, GSR cells displayed higher level of p-MET and p-EGFR than MV522 cells (Fig. 3A), which was confirmed by Western blotting (Fig. 3B and Supplementary Fig. S1A). Despite overexpression of p-MET in GSR cells, a selective MET inhibitor, SU11274, did not show significant antitumor activity as a single agent or in combination with GSK2118436 (Supplementary Fig. S1B). Therefore, EGFR activation seemed to be a major mechanism of acquired resistance to GSK2118436 in GSR cells. Compared with MV522 cells, GSR clones showed greater phosphorylation of EGFR (Y1068) but unchanged p-AKT (S473; Fig. 3B). Notably, GSR clones showed greater levels of GTP-bound RAS, suggesting that activated EGFR stimulates RAS-GTP formation and promotes downstream signaling through CRAF activation (Fig. 3C).

On the basis of constitutive EGFR activation, we reasoned that upregulation of EGFR ligands could contribute to resistance to GSK2118436 in GSR cells. Compared with MV522 cells, the mRNA expression levels of several EGFR ligands, including TGFα, epiregulin, amphiregulin, and heparin-binding EGF (HB-EGF), were significantly increased in GSR clones (Supplementary Fig. S2A). Among the ligands, HB-EGF was the most significantly upregulated in GSR cells (~7-fold). In addition, HB-EGF treatment rescued GSK2118436-induced suppression of p-ERK1/2, but not at all p-MEK1/2 in MV522 cells (Supplementary Fig. S2B). Suppression of p-ERK1/2 was also rescued by HB-EGF stimulation in HCC364 cells (Supplementary Fig. S2B). We observed no effect of other ligands on ERK1/2 activation. Consistent with these effects on signaling, treatment with HB-EGF, but not the others, dramatically enhanced MV522 cell viability in the presence of GSK2118436 (Fig. 3D), indicating that HB-EGF–mediated hyperactivation of EGFR might promote resistance to BRAF inhibition. Indeed, only HB-EGF treatment exerted strong protective effects against GSK2118436, as assessed by a colony formation assay (Fig. 3E). GSK2118436-induced suppression of p-ERK1/2 and enhanced cell viability mediated by HB-EGF treatment were not observed in BRAF V600E–mutant A375 melanoma cell line (Supplementary Fig. S3A and S3B).

Together, our results suggest that autocrine HB-EGF–EGFR signaling promotes acquired resistance to GSK2118436 via EGFR-dependent RAS activation.

Inhibition of EGFR restores sensitivity to GSK2118436 by suppressing RAS-mediated CRAF transactivation in GSR clones

Next, we determined whether combined inhibition of EGFR could restore sensitivity to GSK2118436 in GSR cells. In cell viability assays, the GSR clones were insensitive to gefitinib (an EGFR TKI) or cetuximab (an anti-EGFR mAb) monotherapy (Fig. 4A). Notably, combination of either gefitinib or cetuximab with GSK2118436 showed significant synergistic antitumor effects in MTT assays and colony formation assays (Fig. 4A and B). We used a GTP-RAS activity assay and Western blot analysis to measure the activation status of EGFR–RAS–RAF–MAPK signaling

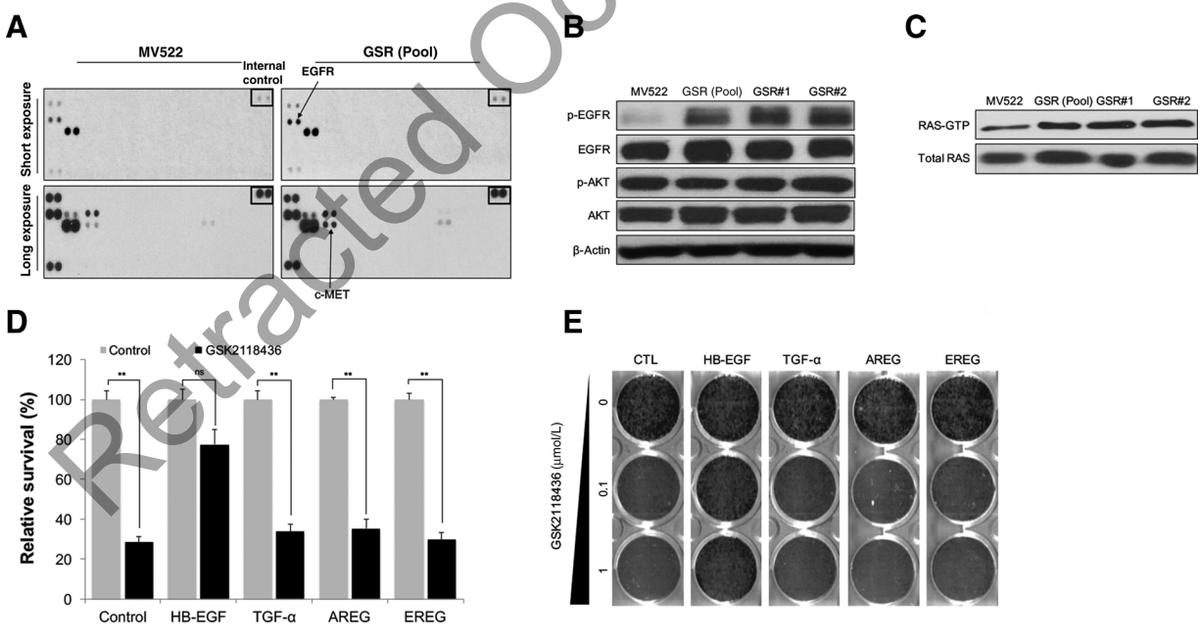

Figure 3.

Activation of EGFR–RAS signaling confers resistance to GSK2118436 in GSR cells. A, phospho-RTK arrays showed activation of EGFR and c-MET in GSR cells. B, cellular lysates were probed with the indicated antibodies in Western blots. C, GTP-bound RASs were measured by a RAS-GTP pulldown assay in all cells. D, in MTT assays, MV522 cells were treated in control or with 1 μmol/L GSK2118436 after treatment with each ligand. E, colony formation assay of MV522 cells after cotreatment of each ligand and GSK2118436 at the indicated concentration for 14 days. All cells were fixed, stained with crystal violet (0.005%), and photographed. All data are normalized to MV522 or control and are displayed as mean ± SEM ($n = 3$; **, $P < 0.01$; ns, not significant).

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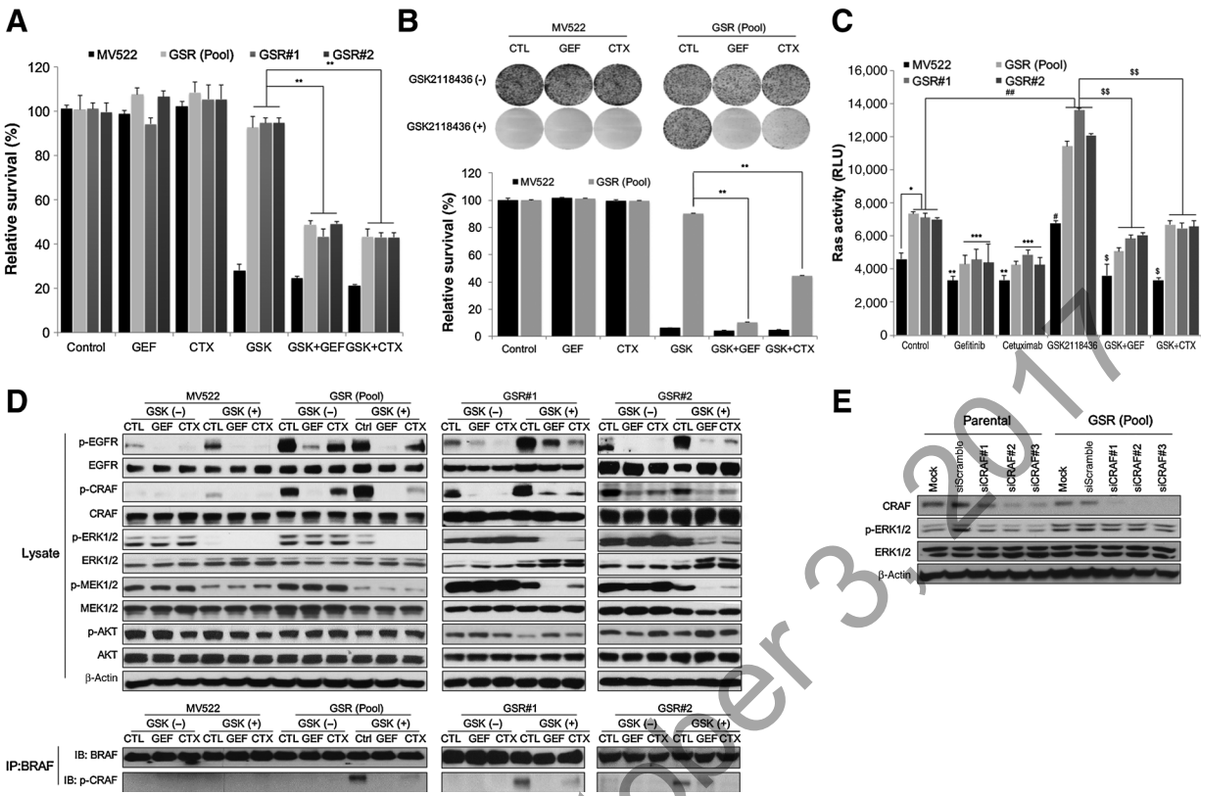

Figure 4.

Inhibition of EGFR restores sensitivity to GSK2118436 by suppressing RAS-mediated CRAF transactivation in GSR clones. A, cells were treated in control or with 1 μmol/L gefitinib (GEF), 1 μmol/L cetuximab (CTX), or 0.1 μmol/L GSK2118436 (GSK), alone or in combination for 72 hours, and viable cells were determined by MTT assay. B, in colony formation assay, cells were fixed, stained, and photographed after treatment with the indicated inhibitors at the same concentration as in Fig. 5A for 14 days. Graphs, quantification of colony. All data are normalized to GSR cells with GSK2118436 treatment and are displayed as mean ± SEM ($n = 3$; **, $P < 0.01$). C, cells were treated with the indicated inhibitor alone or in combination for 24 hours, and levels of active GTP-bound RAS were determined by a RAS-GTP ELISA assay. Error bars, mean ± SEM ($n = 3$). *, $P < 0.05$, GSR cells versus MV522 cells in control; **, $P < 0.01$; ***, $P < 0.001$, control versus gefitinib or cetuximab in MV522 cells and GSR cells; #, $P < 0.05$; ##, $P < 0.01$, control versus GSK2118436 in MV522 cells and GSR cells; $, $P < 0.05$; $$, $P < 0.05$, GSK2118436 versus combination of GSK2118436 with gefitinib or cetuximab in MV522 cells and GSR cells, respectively. D, lysates were evaluated by Western blots with the indicated antibodies, and endogenous BRAF was immunoprecipitated (IP), washed, and immunoblotted (IB) for p-CRAF. E, cells were transfected for 72 hours with scrambled siRNA or CRAF siRNA, respectively. Lysates were probed with the indicated antibodies.

components in MV522 cells and GSR clones during treatment with GSK2118436 alone or in combination with gefitinib or cetuximab (Fig. 4C and D). The baseline RAS activity in the absence of GSK2118436 was significantly higher in GSR clones than MV522 cells ($P < 0.05$). Furthermore, RAS activities in both GSR and MV522 cells treated with GSK2118436 were enhanced over the baseline control, which was significantly more pronounced in GSR cells (75% increase in GSR vs. 45% increase in MV522, $P < 0.01$). Importantly, rebound increase in the RAS activities upon GSK2118436 in both GSR and MV522 cells were abrogated by combining GSK2118436 with gefitinib or cetuximab, which was also significantly more pronounced in GSR cells (65% reduction in GSR vs. 35% reduction in MV522, $P < 0.01$), suggesting a dominant role of EGFR on RAS activation in GSR cells.

Furthermore, when compared with GSK2118436 or EGFR inhibitor monotherapy, the combination of GSK2118436 with either gefitinib or cetuximab markedly reduced the levels of p-EGFR, p-CRAF, and p-ERK1/2 in GSR clones. Particularly, cotreatment of GSK2118436 and gefitinib inhibited p-ERK1/2 almost completely in all GSR clones, similar to the effects of GSK2118436 treatment in MV522 cells. GSK2118436 or EGFR inhibitor monotherapy could not efficiently abolish activation of EGFR–CRAF–ERK signaling in GSR clones (Fig. 4D). EGFR inhibitor treatment, either alone or in combination with GSK2118436, did not decrease the level of p-AKT in GSR clones, indicating that EGFR activation might not contribute to AKT phosphorylation in these resistant cells (Fig. 4D). p-EGFR and RAS-GTP were increased in MV522 cells, likely due to feedback activation of EGFR signaling upon MAPK pathway inhibition (Fig. 4C and D).

Next, we evaluated whether the increased amount of p-CRAF directly activated ERK1/2 in GSR cells. Knockdown of CRAF by siRNA did not affect ERK activity in GSR cells, suggesting that activated CRAF may not be directly responsible for activation of ERK1/2 (Fig. 4E).

RAF inhibitors were reported to transactivate RAF dimers and enhance ERK1/2 signaling in *BRAF* wild-type melanoma, and RAF inhibitor–induced RAF dimerization is RAS dependent (17). Given the high level of GTP-bound RAS and activated CRAF in GSR clones, we sought to determine whether GSK2118436

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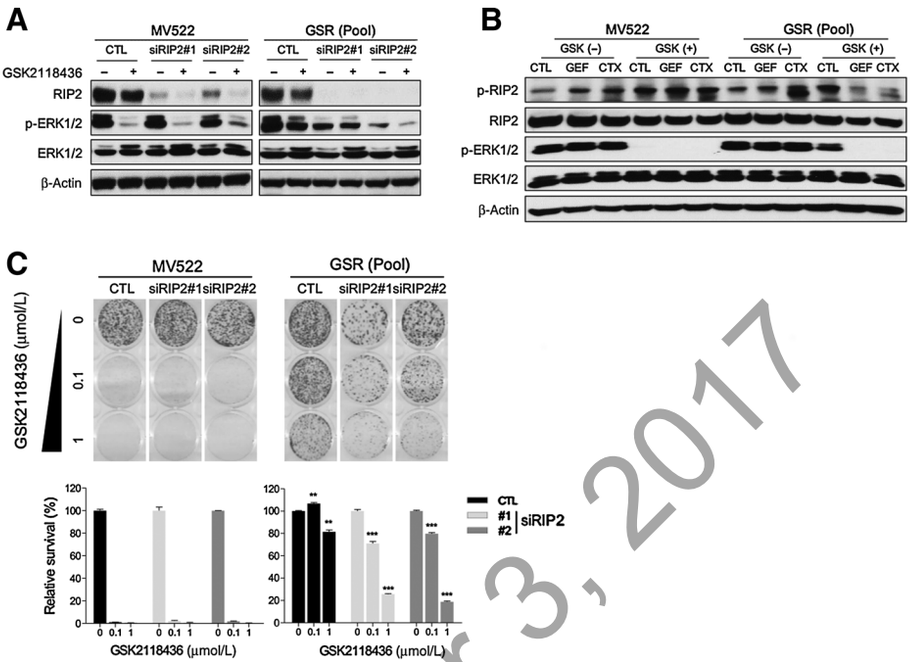

Figure 5.

Activation of RIP2 is responsible for activation of ERK in GSR cells. A, cells were transfected for 24 hours with scrambled siRNA and RIP2 siRNA, respectively, and treated with or without GSK2118436 for 48 hours. Lysates were probed with the indicated antibodies. B, cells were treated with gefitinib (GEF) or cetuximab (CTX) in the absence or presence of GSK2118436. Lysates were probed with the indicated antibodies. CTL, control. C, cells were transfected for 24 hours with scrambled siRNA or RIP2 siRNA, respectively, and treated with or without GSK2118436 at the indicated concentration for 14 days before staining with crystal violet. Data are normalized to GSR cells with GSK2118436 treatment and are displayed as mean ± SEM ($n = 3$; **, $P < 0.01$; ***, $P < 0.001$).

promoted BRAF-CRAF heterodimer via an immunoprecipitation assay. Indeed, as in *BRAF* wild-type melanoma, treatment with GSK2118436, but not gefitinib or cetuximab, induced interaction between BRAF and CRAF in GSR clones (Fig. 4D). Moreover, interaction between BRAF and CRAF disappeared upon combination treatment of GSK2118436 with gefitinib or cetuximab in GSR clones.

Together, these data indicate that GSK2118436-induced formation of BRAF-CRAF heterodimers and CRAF transactivation, which is dependent on EGFR-mediated RAS activation, is responsible for sustained ERK1/2 activation in GSR cells.

Receptor-interacting protein-2 kinase is partly responsible for sustained ERK1/2 activation in GSR clones

Receptor-interacting protein (RIP) kinases, which consist of seven members of serine/threonine kinases (RIP 1–7), have emerged as essential sensors of cellular stress (18). In response to various extracellular and intracellular stresses, RIP kinases play essential roles in prosurvival, inflammatory, and immune responses and in proapoptotic processes. Particularly, RIP2 kinase mediates the activation of MAPKs, such as p38, JNK, and ERK1/2 (19).

In GSR clones, ERK1/2 activation was independent of MEK1/2, which was supported by GSK2118436-mediated suppression of MEK1/2, but not ERK1/2. Thus, we evaluated whether inhibition of RIP2 by siRIP2 regulates ERK1/2 activation. Knockdown of RIP2 suppressed p-ERK1/2 in GSR cells, but not in MV522 cells (Fig. 5A).

Given that the combination of GSK2118436 and EGFR inhibitors suppressed p-ERK1/2 by inhibiting EGFR/RAS/CRAF signaling (Fig. 4D), we evaluated whether the combination treatment also regulates p-RIP2. Compared with GSK2118436 or EGFR inhibitor monotherapy, the combination of GSK2118436 with either gefitinib or cetuximab reduced the level of p-RIP2, accompanied by inactivation of ERK1/2, only in GSR cells (Fig. 5B). Next, we examined whether RIP2 inhibition can overcome acquired resistance to GSK2118436 in GSR cells. Consistent with the effects of RIP2 on ERK1/2 activation, the antiproliferative effect of GSK2118436 in GSR cells was increased by cotreatment of siRIP2 in colony formation assays (Fig. 5C). Overexpression of RIP2 wild type or RIP2 (S176E; which is phosphorylated form) induces the resistance to GSK2118436 in MV522 cells compared with EGFP-C1 vector expressing MV522 cells via retained phosphorylation of ERK1/2 (Supplementary Fig. S4). Furthermore, RIP2 silencing modestly decreased the mRNA expression of HB-EGF, but not of the other EGFR ligands in GSR cells (Supplementary Fig. S5).

Together, these results suggest that RIP2 activation may contribute to the sustained activation of ERK1/2 in GSR cells and combined inhibition of BRAF and RIP2 could partly overcome acquired resistance to GSK2118436.

Inhibition of EGFR signaling potentiates the antitumor activity of GSK2118436 in GSR xenograft models

To further examine whether combined BRAF and EGFR inhibition can overcome acquired resistance to GSK2118436 *in vivo*, we tested the effects of GSK2118436, gefitinib, and their combination on the growth of GSR xenograft tumors established in nude mice. The combined treatment of GSK2118436 and gefitinib showed a strong synergistic inhibition of tumor growth compared with controls ($P < 0.01$) or with treatment with GSK2118436 ($P < 0.01$) or gefitinib alone ($P < 0.01$; Fig. 6A) in GSR xenografts but not in MV522 xenografts. The synergistic antitumor effect of combined GSK2118436 and gefitinib treatment was also detected by immunohistochemical staining for Ki67 (a marker for cell proliferation), p-EGFR, p-CRAF, p-RIP2, and p-ERK1/2 (Fig. 6B). Consistent with *in vitro* observations, staining for Ki67, p-EGFR, p-CRAF, p-RIP2, and p-ERK1/2 was markedly reduced upon combined administration of GSK21128436 and gefitinib only in GSR xenografts compared with controls or treatment with GSK2118436 or gefitinib alone. These results support combined inhibition of

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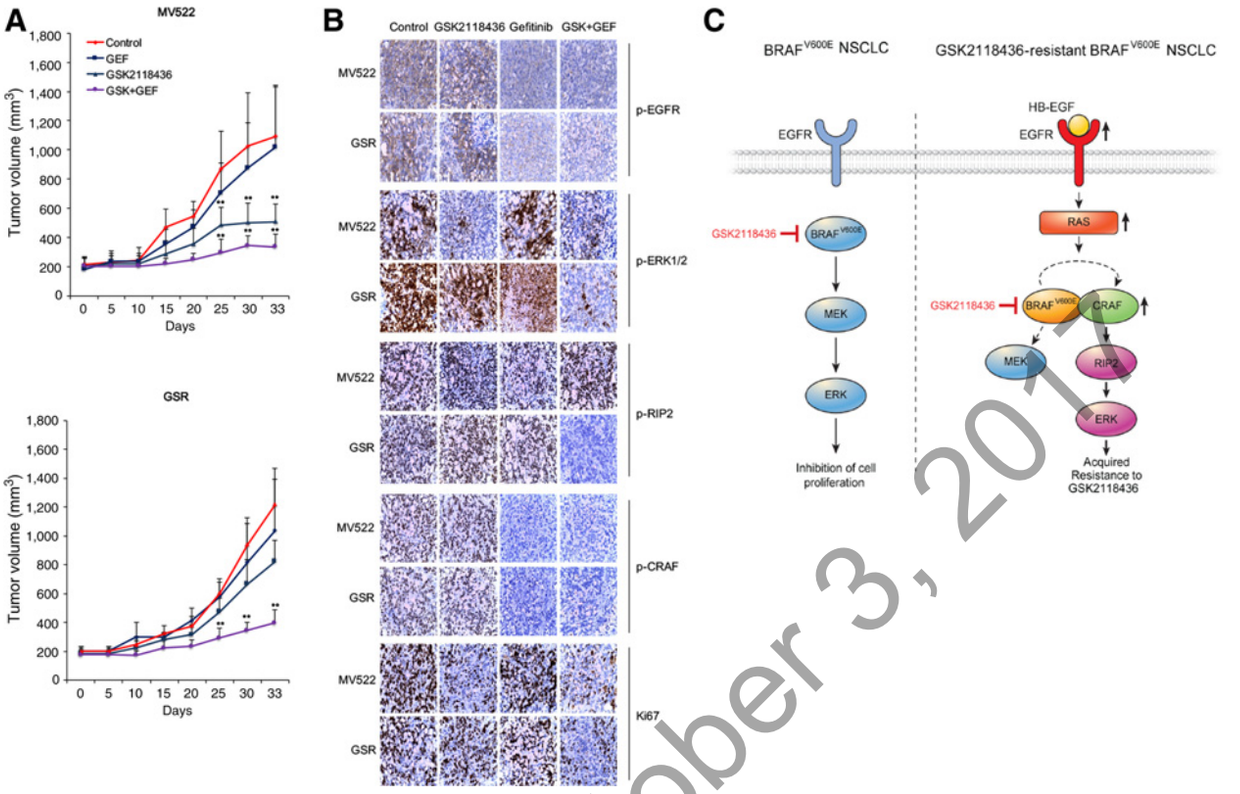

Figure 6.

Combination of GSK2118436 and gefitinib leads to enhanced *in vivo* efficacy in GSR (pool) cells. A, xenografts derived from MV522 cells and GSR (pool) cells were treated with vehicle (control), GSK2118436 (GSK, 300 mpk daily), or gefitinib (GEF, 50 mpk daily) alone or in combination (GSK + GEF) for 33 days. Inhibitor treatments commenced when tumor volume reached 150 mm^3, and mean tumor volumes shown (±SEM) are normalized to control ($n = 7$ mice/group; **, $P < 0.01$). B, tumors were harvested 6 hours after administration of the final dose and evaluated by IHC for p-EGFR, p-ERK1/2, p-RIP2, p-CRAF, and a marker of cell proliferation (Ki67). Representative examples are shown. C, the mechanism of acquired resistance to GSK2118436 in NSCLC^{V600E}. In MV522 cells, BRAF controls cell growth and proliferation via MEK–ERK signaling. GSK2118436 effectively inhibits cell growth via the MEK-ERK pathway (left). However, GSR cells show diminished dependence on BRAF–MEK–ERK signaling. Activation of EGFR causes increasing RAS activity in the presence of GSK2118436 and promotes CRAF transactivation by dimerization with BRAF. Activation of RIP2 in these resistant cells is caused by both BRAF inhibition and EGFR–RAS–CRAF activation, and activated RIP2 sustains cell growth by reactivating ERK1/2 in the presence of GSK2118436 (right).

BRAF and EGFR as a promising therapeutic strategy for NSCLC^{V600E} with acquired resistance to GSK2118436.

Discussion

In our study, we found that sustained ERK1/2 activation via EGFR-mediated activation of RAS and CRAF confers acquired resistance to GSK2118436 in NSCLC^{V600E}. Upregulation of the EGFR ligand, HB-EGF, and autocrine EGFR activation leads to constitutive activation of the canonical downstream cascade of EGFR signaling and potentiates resistance to GSK2118436. The most interesting and novel finding of our study is that ERK1/2 activation, the critical contributor to BRAF inhibitor resistance, is independent of MEK1/2 activity but partly dependent on RIP2 kinase activity, which distinguishes BRAF inhibitor–resistant NSCLC^{V600E} from BRAF inhibitor–resistant melanoma^{V600E}.

To our knowledge, various acquired resistance mechanisms to BRAF inhibitors have been reported to date (20): alternative splicing of BRAF (14), BRAF amplification (21), CRAF overexpression (22), and activation of EGFR signaling (15, 23, 24). In the former mechanism reported in melanoma^{V600E}, p61 splicing variant (p61BRAF)–driven resistance to BRAF inhibitors reactivated MEK–ERK signaling through enhanced dimerization of the aberrant BRAF isoform (14). The latter mechanism occurred through enhanced expression of EGFR ligands that potentiated autocrine EGFR activation, which promoted both MEK-dependent ERK reactivation and AKT1/2 activation (25), in contrast to MEK-independent ERK reactivation in our study. Accordingly, treatment of BRAF inhibitor–resistant cells with erlotinib either alone or in combination with vemurafenib decreased the level of p-AKT1/2 in the study, indicating that EGFR activation critically contributed to AKT1/2 activation (25), whereas treatment of GSR cells with an EGFR inhibitor alone or in combination with GSK2118436 herein did not decrease the level of p-AKT1/2 despite significant reduction of p-EGFR (Fig. 4D).

Contrary to the previous study (21), the levels of p-EGFR, but not p-AKT1/2, were different between BRAF inhibitor–sensitive and -resistant cells in our study (Figs. 3B and 4D), suggesting that increased p-EGFR levels could be a biomarker of BRAF inhibitor resistance acquisition in NSCLC^{V600E}. Furthermore, the level of

p-EGFR may contribute to distinguish BRAF inhibitor–resistant NSCLC^{V600E} with EGFR dependence from that with MAPK reactivation by other mechanisms, such as mutation of the MAPK pathway genes. Discrimination between at least these two mechanisms will be important for developing treatment strategies to overcome resistance to BRAF inhibitors. For example, as in melanoma^{V600E}, p61BRAF-driven MAPK pathway reactivation can be subverted by combined BRAF and MEK inhibition (16). A current clinical trial is testing whether a combination of GSK2118436 and trametinib (GSK1120212) can delay or prevent acquired drug resistance in patients with NSCLC^{V600E} (NCT01336634). However, our *in vitro* and *in vivo* data clearly suggest that a subset of BRAF inhibitor–resistant NSCLC^{V600E} patients with EGFR-mediated MAPK pathway reactivation might benefit most from combined inhibition of BRAF and EGFR. Importantly, nearly complete inhibition of p-ERK1/2, which is an essential prerequisite for tumor responses to BRAF inhibitor, and significantly reduced GTP-bound RAS and p-CRAF were observed only after combined treatment with GSK2118436 and EGFR inhibitors. MEK-independent activation of ERK1/2 and lack of synergistic antitumor effects with cotreatment of GSK2118436 and AZD6244 (Fig. 2C) does not support further clinical investigation of combined BRAF and MEK inhibition in this subset of NSCLC^{V600E}.

As in *BRAF* wild-type melanoma (17), GSK2118436 enhanced BRAF–CRAF dimerization and transactivated CRAF, resulting in paradoxical activation of ERK1/2, and drug-mediated transactivation of RAF dimers was dependent on RAS activity. Indeed, there was a significant recovery of p-EGFR following GSK2118436 treatment in GSR cells (Fig. 4D), likely due to feedback activation of EGFR caused by BRAF inhibition (26). Furthermore, in addition to higher baseline RAS activity, GSR cells exhibited far greater enhancement of RAS activity after treatment with GSK2118436 than MV522 cells. Given the lack of *RAS* mutation and inhibition of RAS activity with EGFR inhibitor treatment either alone or in combination with GSK2118436 in GSR cells, we attributed RAS activation to autocrine activation of EGFR in our resistant model. Importantly, the maximum enhancement of EGFR and RAS activity in the presence of GSK2118436 might contribute to efficient formation of BRAF-CRAF heterodimers, ultimately leading to sustained ERK activation and drug resistance. In contrast, enhancement of RAS activity was less pronounced in MV522 cells than in GSR cells, resulting in dissociation of the BRAF–CRAF complex and, therefore, minimal activation of CRAF.

Most interestingly, we found that sustained activation of ERK1/2 could be partly dependent on RIP2 activity, but not on MEK activity, although RIP2 activation is not the major determinant of ERK1/2 activation. To our knowledge, this is the first report on the mechanism of acquired resistance to BRAF inhibitors describing MEK-independent ERK reactivation in any type of BRAF V600E–mutant cancers. RIP2 is a serine/threonine kinase that serves as an intracellular mediator of TNF receptor-α response to assemble a signaling complex leading to apoptosis (27, 28). In an earlier report, RAS-activated CRAF phosphorylated and activated RIP2 kinase, which then activated ERK1/2 (29). Herein, we assumed that RIP2 activation was likely attributable to CRAF activation driven by EGFR-mediated RAS activity based on abrogation of RIP2 activation only after combined treatment with GSK2118436 and EGFR inhibitor. Finally, the combination of GSK2118436 and siRIP2 suppress RIP2-mediated ERK reactivation and cell growth, supporting a role for RIP2 as a contributor to the acquired resistance to GSK21128436 in GSR cells.

On the basis of this unique mechanism of ERK1/2 reactivation and its potential druggability, our results suggest that a combination of BRAF inhibitor and EGFR inhibitor may be a promising therapeutic strategy for patients with NSCLC^{V600E} with acquired resistance to BRAF inhibitors.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Authors' Contributions

Conception and design: K.W. Jang, H.R. Kim, S. Paik, B.C. Cho
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Molecular Cancer Therapeutics

Retraction: EGFR-Mediated Reactivation of MAPK Signaling Induces Acquired Resistance to GSK2118436 in BRAF V600E–Mutant NSCLC Cell Lines

This study (1) makes extensive use of a purported BRAF-V600E–driven non-small cell lung cancer (NSCLC) cell line, MV522. However, ATCC discontinued offering the cell line in July, 2012 because "STR yielded similar profiles for MV522 (ATCC CRL-2519) and HT-29 (ATCC HTB-38)" (2). The International Cell Line Authentication Committee (ICLAC) lists MV522 as a cell line for which no authentic stock is known (3). The ICLAC states that MV522 cells are not of lung cancer origin but are a cross contamination of the HT-29 BRAF-V600E–driven colorectal cancer cell line. Because the claims of the article rest on the cell line being non–small cell lung cancer cells, the article is being retracted by the editors.

The authors have been informed of this decision.

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EGFR-Mediated Reactivation of MAPK Signaling Induces Acquired Resistance to GSK2118436 in BRAF V600E–Mutant NSCLC Cell Lines

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