Impaired SHP2-Mediated Extracellular Signal-Regulated Kinase Activation Contributes to Gefitinib Sensitivity of Lung Cancer Cells with Epidermal Growth Factor Receptor–Activating Mutations

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Abstract

Most non–small cell lung cancers (NSCLC) display elevated expression of epidermal growth factor receptor (EGFR), but response to EGFR kinase inhibitors is predominantly limited to NSCLC harboring EGFR-activating mutations. These mutations are associated with increased activity of survival pathways, including phosphatidylinositol 3-kinase/AKT and signal transducer and activator of transcription 3/5. We report that EGFR-activating mutations also surprisingly lead to decreased ability to activate extracellular signal-regulated kinase (ERK) compared with wild-type EGFR. In NSCLC cells and mouse embryonic fibroblasts expressing mutant EGFR, this effect on ERK correlates with decreased EGFR internalization and reduced phosphorylation of SHP2, a tyrosine phosphatase required for the full activation of ERK. We further show that ERK activation levels affect cellular response to gefitinib. NSCLC cells with EGFR mutation display reduced gefitinib sensitivity when ERK activation is augmented by expression of constitutively active mutants of mitogen-activated protein kinase/ERK kinase (MEK). Conversely, in a NSCLC cell line expressing wild-type EGFR, gefitinib treatment along with or following MEK inhibition increases death response compared with treatment with gefitinib alone. Our results show that EGFR-activating mutations may promote some survival pathways but simultaneously impair others. This multivariate alteration of the network governing cellular response to gefitinib, which we term “oncogene imbalance,” portends a potentially broader ability to treat gefitinib-resistant NSCLC. Cancer Res; 70(9); 3843–50. ©2010 AACR.

Introduction

Up to 80% of non–small cell lung cancers (NSCLC) display elevated expression of epidermal growth factor receptor (EGFR; ref. 1), but response to EGFR kinase inhibitors is predominantly limited to the 10% to 20% of tumors that harbor somatic EGFR-activating mutations (2–6). Consistent with patient response data, NSCLC cell lines bearing EGFR-activating mutations display enhanced sensitivity to gefitinib and erlotinib in culture (3, 6–8). Studies of these cell lines show increased basal phosphorylation of EGFR and increased activity of survival-associated signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/AKT and signal transducer and activator of transcription 3/5 (STAT3/5; refs. 3, 7, 9). Exposure of mutant-bearing NSCLC cells to EGF results in more protracted increases in EGFR phosphorylation than for NSCLC cells expressing wild-type EGFR (3), consistent with the reduced endocytosis of EGFR mutants (10, 11). Elevated expression of ERBB3 has been observed in some gefitinib-sensitive cells, including those lacking EGFR mutation (7, 12). ERBB3 phosphorylation is apparently promoted by EGF in these cells and is the primary mediator of PI3K/AKT activity (7).

EGFR-activating mutations also perturb gefitinib and ATP binding and promote receptor inhibition at lower gefitinib concentrations (3, 13, 14). This effect propagates to multiple signaling pathways and in some cases may involve differential inhibition of ERBB3. For example, the phosphorylation of AKT and ERBB3 is equally or more responsive to gefitinib than EGFR phosphorylation in certain gefitinib-sensitive cell lines (7). Activating mutations may also result in signaling after EGF inhibition that favors apoptosis over survival (15). Each of these features may partially

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explain increased sensitivity to gefitinib of cells with EGFR-activating mutations.

In tumors that are initially responsive to gefitinib or erlotinib, resistance mechanisms may arise that abrogate inhibition of survival signaling. In ∼50% of acquired resistance cases, a secondary T790M mutation arises that disfavors the binding of gefitinib to EGFR (14, 16, 17). In other cases, MET amplification maintains ERBB3/P38K/AKT activity after treatment with gefitinib (12). Mutations of BRAF and KRAS also correlate with primary resistance to gefitinib (18–21).

Because these mutations increase EGFR kinase activity (22), the finding that the activities of some downstream survival pathways are elevated is not surprising. These alterations apparently lead to cellular dependence on EGFR, or oncogene addiction, as shown by the finding that EGFR inhibition or knockdown leads to apoptosis in NSCLC cells expressing mutant, but not wild-type, EGFR. How elevated survival signaling leads to EGFR dependence, however, remains poorly understood.

We report that activation of extracellular signal-regulated kinase (ERK) is impaired by the expression of EGFR mutants compared with wild-type. Reduced EGFR-elicited activation of ERK in mutant EGFR-bearing cells correlates with diminished EGFR internalization and reduced phosphorylation of the protein tyrosine phosphatase SHP2, a positive regulator of ERK activity (23). Moreover, the effect on SHP2 phosphorylation is linked to defective EGFR internalization. We further show that ERK activity affects cellular response to gefitinib. NSCLC cells expressing an EGFR mutant exhibit reduced death response to gefitinib when ERK activation is augmented by constitutively active mitogen-activated protein kinase/ERK kinase (MEK). Conversely, NSCLC cells expressing wild-type EGFR are more sensitive to gefitinib when cotreated or pretreated with the MEK inhibitor U0126. Our results suggest that EGFR-activating mutations are associated with enhancement of some survival signals but impairment of others, with the integrated effects influencing cellular response to gefitinib.

Materials and Methods

Cells. Wild-type Egfr homozygous (EgfrWT/WT), L858R homozygous (EgfrL858R/L858R), and heterozygous (EgfrWT/L858R) primary mouse embryonic fibroblasts (MEF) were created as described elsewhere.7 3T3-immortalized MEFs expressing CreER and with one truncated (null) Shp2 allele and one intact allele with a section of exon 11 flanked by LoxP sites (denoted Shp2fl/−) were created as described elsewhere.8 H1666 (obtained from the American Type Culture Collection) and H3255 (Dr. Pasii Janne, Dana-Farber Cancer Institute, Boston, MA; functionally characterized by gefitinib sensitivity, as described herein) cells were grown in ALC14. HeLa cells with conditional expression of wild-type or K44A dynamin (Dr. Sandra Schmid, Scripps Research Institute, La Jolla, CA; functionally characterized as described herein) were cultured as described elsewhere (24, 25). Primary MEFs and Shp2fl/− MEFs were grown in DMEM. All media contained 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine, and 10% fetal bovine serum (FBS). Shp2fl/− MEFs were cultured in 1 mmol/L 4-hydroxatamoxifen (4-OHT) for 24 hours to induce Shp2 deletion and returned to media without 4-OHT for 36 hours before experiments. Otherwise, cells were plated in six-well dishes and grown for 24 to 48 hours before serum starving (in media containing 0.1% FBS for 12–16 h) or treatment with inhibitors.

Egfr expression. Wild-type and L858R Egfr cDNA was generated from mRNA isolated from EgfrWT/WT and EgfrL858R/L858R mouse lines, respectively, and inserted into pMSCV expression vectors with puromycin or hygromycin resistance. Plasmids were transfected into ecotropic Phoenix cells (Dr. Gary Nolan, Stanford University, Stanford, CA), and viral supernatants were used to infect EgfrWT/WT and Shp2fl/− MEFs. Target cells were selected in 2 μg/mL puromycin or 200 μg/mL hygromycin.

MEKDD expression. VSV-G pseudotyped retrovirus was produced by transfecting HEK 293FT cells with pBABE-puro-MEK1DD (26) or pBABEpuro-MEK2DD (27) and the packaging plasmids pMD-G and pMD-p/Δ. Virus was harvested 48 and 72 hours after transfection, concentrated by ultracentrifugation, and used to infect H3255 cells. Target cells were selected in 2 μg/mL puromycin.

Immunoblotting. Lysates were prepared in a standard buffer containing detergents, buffer salts, and protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 4°C and 13,200 rpm for 10 minutes, and protein concentration was determined by micro–bicinchoninic acid assay. Denatured and reduced protein was loaded on 10% polyacrylamide gels (20 μg per lane) and transferred to 0.2-μm nitrocellulose. Membranes were blocked in Odyssey blocking buffer (LI-COR), and all antibodies were used according to the manufacturers’ recommendations. Where needed, blots were stripped with 0.2 N NaOH.

Antibodies. Antibodies for EGFR, EGFR pY1068, ERK, ERK pT202/Y204, AKT pS473, SHP2 pY542, SHP2 pY580, MEK1/2, and MEK1/2 pS217/S221 were purchased from Cell Signaling Technology. Antibodies for human and mouse Shp2 were purchased from Epitomics and Santa Cruz Biotechnology, respectively. The glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Calbiochem. IR dye–conjugated secondary antibodies were purchased from Rockland Immunochemicals.

Other reagents. Gefitinib and U0126 were purchased from WuXi Pharmatech and Promega, respectively. Human EGF and platelet-derived growth factor (PDGF) were purchased from PeproTech. Puromycin, propidium iodide (PI), and 4-OHT were purchased from Sigma. Hygromycin B was purchased from Clontech, FBS, penicillin/streptomycin, L-glutamine, geneticin, and all media were purchased from Invitrogen.

EGFR internalization assay. Rate constants for the endocytosis of 125I-EGF (k dissociation) were measured as described previously (28, 29).
**Results**

Previous data show that ERK phosphorylation may be reduced in cells expressing EGFR mutants compared with counterparts expressing wild-type EGFR (EGFRWT; refs. 3, 30), but subsequent studies have focused mainly on the enhancement of pathways including PI3K/AKT and STAT3/5 (3, 7, 9). To explore the possibility that ERK phosphorylation is reduced in the context of EGFR-activating mutations relative to EGFRWT, we compared the phosphorylation of EGFR, AKT, and ERK in the human NSCLC cell lines H1666 (EGFRWT) and H3255 (EGFRR858L) stimulated with 10 ng/mL EGF for up to 1 hour. We chose H3255 for comparison because it is exclusively sensitive to gefitinib (3, 7) and because it carries EGFRR858L, one of the most common NSCLC-associated EGFR mutations (6, 31–33). Western blot analysis is shown in Fig. 1 and Supplementary Fig. S1. As expected, EGFR phosphorylation, monitored at Y1068, was basally elevated and prolonged in response to EGF in H3255 cells compared with H1666. Consistent with previous observations (3, 7), the phosphorylation of AKT at S473 was basally elevated in H3255 cells, and AKT phosphorylation actually decreased in response to EGF. In H1666 cells, AKT phosphorylation increased in response to EGF and returned to baseline levels within 60 minutes. The phosphorylation of ERK at T202/Y204 was uniformly lower in H3255 cells than in H1666 cells, with lower basal levels and delayed increases in response to EGF.

H1666 and H3255 lysates were also probed for phosphorylation of the protein tyrosine phosphatase SHP2 at Y542 (Fig. 1). SHP2 is required for complete ERK activation (23), and its phosphorylation at Y542 is required for its normal activity downstream of several growth factor receptors (34). SHP2 Y542 phosphorylation was induced in H1666 cells in response to EGF by 5 minutes, but its phosphorylation was not induced in H3255 cells at any time point. Samples were also probed for SHP2 phosphorylation at Y580, a site of secondary importance for SHP2 regulation (34). An EGF-inducible band at the appropriate molecular weight was observed for H1666 cells but not for H3255 (Supplementary Fig. S2A). EGF-induced SHP2 Y542 phosphorylation was modest in a number of other NSCLC cell lines with EGFR-activating mutations, including PC9, HCC827, and H1975 (Supplementary Fig. S2B). The mutant-bearing, PTEN-null cell line H1650 did exhibit inducible SHP2 Y542 phosphorylation. Experiments using MDA-468 cells, which are PTEN null, with and without PTEN reconstitution, showed no general effect of PTEN status on SHP2 Y542 phosphorylation (Supplementary Fig. S3).

To determine how ERK activity affects H3255 sensitivity to gefitinib, we created H3255 cells expressing constitutively active mutants of MEK, MEK1DD (26), or MEK2DD (27). Interestingly, MEKDD expression slightly decreased basal ERK phosphorylation below that observed in controls (Supplementary Fig. S4A). Treatment with 1 and 10 μmol/L gefitinib for 10 minutes, however, resulted in complete inhibition of ERK phosphorylation in control cells but only partial inhibition in MEKDD-transduced cells (Fig. 2A), suggesting that both MEKDDs were active. To examine the effect of sustained ERK phosphorylation on H3255 sensitivity to gefitinib, we measured cell permeability to PI 72 hours after treatment with gefitinib (Fig. 2B). At 1 μmol/L gefitinib, the fraction of dead (Fig. 2B) and floating (Supplementary Fig. S4B) cells increased significantly above baseline for control cells but not for either MEKDD-expressing line. At higher gefitinib concentrations, PI permeability of control cells increased monotonically but remained at approximately baseline levels for MEKDD-expressing cells. Although proliferation was obviously impeded by gefitinib in MEKDD-expressing cells, significant numbers of those cells remained adherent even at 10 μmol/L gefitinib.

To assess the potential for increasing cellular sensitivity of NSCLC cells expressing wild-type EGFR to gefitinib through the manipulation of ERK phosphorylation, we measured the response of H1666 cells to treatment with gefitinib alone or in combination with the MEK inhibitor U0126. Thirty-minute exposure of H1666 cells to concentrations of U0126 (≥1 μmol/L) resulted in complete inhibition of ERK phosphorylation (Supplementary Fig. S5A). Treatment of H1666 cells with gefitinib alone (1 or 10 μmol/L) for 5 and 6 days resulted in little to no increase in the fraction of PI-positive cells versus treatment with DMSO. Response to 10 μmol/L U0126 was similarly modest (Fig. 3A and B). When gefitinib was combined with U0126, PI permeability 5 and 6 days after treatment increased beyond that observed for treatment with

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**PI permeability.** Cells were plated in six-well dishes, grown for 24 to 72 hours, and switched to complete media containing appropriate concentrations of gefitinib, U0126, and DMSO. After an additional 3 to 6 days, adherent and floating cells were pooled and resuspended in PBS-PI. Flow cytometry data were acquired on a Becton Dickinson FACSCalibur and analyzed using FlowJo.
gefitinib or U0126 alone, especially for 10 μmol/L gefitinib (Fig. 3A and B). Microscopy revealed a high percentage of floating cells for combination treatments (Supplementary Fig. S5B). To attempt to mimic the condition of chronic low-level ERK phosphorylation encountered in H3255 cells, H1666 cells were cultured in the presence or absence of 10 μmol/L U0126 for 3 weeks and subsequently treated for 72 hours with DMSO, 1 μmol/L gefitinib, 10 μmol/L U0126, or 1 μmol/L gefitinib and 10 μmol/L U0126 in combination (Fig. 3C; Supplementary Fig. S5C). Control cells that had been maintained in DMSO showed no increase in PI permeability in response to 1 μmol/L gefitinib alone and only a modest increase in response to 1 μmol/L gefitinib in combination with 10 μmol/L U0126. In contrast, for cells that had been cultured in 10 μmol/L U0126, PI permeability increased >2-fold above control for treatment with 1 μmol/L gefitinib and >5-fold above control for treatment with 1 μmol/L gefitinib and 10 μmol/L U0126.

We attempted to recapitulate the ERK and SHP2 phosphorylation trends observed in NSCLC lines in primary MEFs. EgfrWT/WT MEFs expressing similar levels of murine wild-type and L858R Egfr were stimulated with 10 ng/mL EGF for up to 1 hour and analyzed by Western blot (Fig. 4A and B; Supplementary Fig. S6A). Phosphorylation of Shp2 Y542 and Erk T202/Y204 was more modest in cells expressing EgfrL858R than in cells expressing EgfrWT, but the differences were not as pronounced as in H1666 and H3255 cells. In contrast to what has been observed in NSCLC cells, Akt phosphorylation was not enhanced in cells expressing EgfrL858R and was actually decreased compared with cells expressing EgfrWT at 60 minutes (Supplementary Fig. S6B).

The correlation between SHP2 and ERK phosphorylation levels in NSCLC cells and MEFs expressing EGFRL858R suggests, but does not guarantee, that SHP2 may be less active in these cells (34). We investigated the functional role of Shp2 in promoting Erk phosphorylation by expressing EgfrWT or EgfrL858R in Shp2fl/fl 3T3-immortalized MEFs and probing for response to EGF for up to 1 hour (Fig. 4C and D; Supplementary Fig. S7A). Shp2fl/fl MEFs constitutively express CreER and contain only one intact Shp2 allele, which has a portion of exon 11 flanked by LoxP recombination sites. As described in Materials and Methods, treatment of these cells with 4-OHT results in loss of Shp2 and its product. For Shp2fl/fl cells not treated with 4-OHT (i.e., cells retaining one functional Shp2 allele), Erk phosphorylation was induced more gradually (similar to vector-transduced control cells) in cells expressing EgfrL858R compared with EgfrWT. When this panel of cells was treated with 4-OHT, induction of Erk phosphorylation was decreased for all three cell types, with the largest deviations from baseline (without 4-OHT treatment) observed for cells transduced with vector and EgfrWT. These
findings suggest that Shp2 plays a more prominent role in promoting Erk phosphorylation downstream of EgfrWT than EgfrL858R. Interestingly, Shp2fl− cells became more responsive to gefitinib after treatment with 4-OHT (Supplementary Fig. S7B), suggesting that impairment of Shp2 activity may promote cellular sensitivity to gefitinib.

Reduced phosphorylation of ERK downstream of EGFRL858R may be linked to the previously established relationship between EGFR endocytosis and ERK activation (25) given that activating mutations of EGFR are endocytosis impaired (10, 11). Rate constants for the endocytosis of 125I-EGF (ke) measured using a well-established technique (28, 29) were previously reported for the NSCLC cell lines A549 (EGFRWT, gefitinib resistant) and PC9 (EGFRdelE746-A750, gefitinib sensitive) as 0.14 and 0.04 minutes−1, respectively (10). Using the same technique, we measured ke values for H1666 and H3255 of 0.21 and 0.02, respectively (Fig. 5A). We found that this trend was reproduced in a panel of EgfrWT/WT, EgfrWT/L858R, and EgfrL858R/L858R primary MEFs, where ke decreased monotonically with increasing relative abundance of the mutant allele (Fig. 5B). Reductions in ke relative to control were also observed when EgfrWT and EgfrL858R were expressed in the EgfrWT/WT background, with the greatest reduction observed for EgfrL858R expression (Fig. 5C). These results indicate that both receptor expression level and mutational status play a role in determining ke. For Shp2fl− cells (without 4-OHT treatment), ke for vector control cells was significantly lower than in the EgfrWT/WT background, but increased Ergr expression resulted in further reduced ke values, with the lowest observed ke for EgfrL858R expression (Fig. 5D). No change was observed in the measured ke for Shp2fl− cells treated with 4-OHT (Supplementary Fig. S7C).

To determine if SHP2 Y542 phosphorylation depends on EGFR endocytosis, we investigated SHP2 phosphorylation in HeLa cells with inducible expression of wild-type and dominant-negative K44A dynamin (DynWT and DynK44A, respectively; ref. 25). Dynamin is a GTPase involved in clathrin-mediated endocytosis of receptor tyrosine kinases, and its ectopic expression is induced in the HeLa cells we used in the absence of tetracycline. DynK44A expression has been previously shown to reduce rates of EGFR endocytosis compared with DynWT (25), a phenotype we confirmed with the 125I-EGF–based assay (Supplementary Fig. S8A). Western blot analysis of these cells treated with 10 ng/mL EGF for up to 1 hour is shown in Fig. 6A and B and Supplementary Fig. S8B. Phosphorylation of EGFR Y1068 was prolonged (Supplementary Fig. S8B) and that of ERK was impaired (Fig. 6B) in cells expressing DynK44A compared with DynWT. Slight reduction in AKT phosphorylation at S473 was also observed with DynK44A expression (Supplementary Fig. S8B). DynK44A expression also resulted in reduced SHP2 Y542 phosphorylation (Fig. 6A), but the effect was more modest than observed in NSCLC lines. Interestingly, a similar SHP2 Y542 phosphorylation trend was observed after stimulation with...
DynK44A HeLa cells cultured with or without tetracycline were treated with up to 20 μmol/L gefitinib for 72 h and analyzed for PI permeability. In the absence of tetracycline, however, cells expressing DynK44A showed increased death response to gefitinib concentrations ≥15 μmol/L.

**Discussion**

Our study shows that the EGFR<sup>L858R</sup> mutant promotes ERK activity less efficiently than EGFR<sup>WT</sup> in human NSCLC cells and MEFs and that this effect is associated with impaired endocytosis of EGFR mutants and concomitant reduction in SHP2 Y542 phosphorylation. Although previous data support the notion that NSCLC-associated EGFR mutants may not promote ERK activity as efficiently as wild-type EGFR (3, 30), this effect had not been pursued. Moreover, although a connection between EGFR internalization and ERK activation had been established (25), a link between this effect and the impairment of mutant EGFR internalization (10, 11) had not been raised. To the best of our knowledge, our present study also represents a first report of differences in the role of SHP2 in promoting ERK activity downstream of wild-type and mutant EGFR.

SHP2 is required for complete activation of ERK (23), and its phosphorylation at Y542 is necessary for its full activity downstream of some, but not all, growth factors. For example, Shp2 Y542 phosphorylation has been found to be less important for mediating Erk activation downstream of Egfr than downstream of Fgfr or Pdgfr in mouse fibroblasts (34). Those studies did not explore the effects of receptor expression levels, however, and it has been argued that such effects may alter the occurrence and relevance of SHP2 Y542 phosphorylation in response to EGF (34). In addition to our finding of differential SHP2 phosphorylation, the finding that Shp2 deletion in MEFs leads to less relative impairment of EGF-mediated Erk activity downstream of Egfr<sup>L858R</sup> than Egfr<sup>WT</sup> suggests that Shp2 activity may be less efficiently promoted by Egfr<sup>L858R</sup>. Thus, ERK activity may be reduced downstream of mutant EGFR expression because of defects in receptor trafficking and the functional role (but possibly not phosphatase activity) of SHP2. Data from HeLa cells with inducible Dyn<sup>WT</sup> or Dyn<sup>K44A</sup> expression indicate that EGFR trafficking and SHP2 phosphorylation are linked.

Further studies indicated that, beyond the apparent link to receptor internalization, the mechanism underlying differential SHP2 involvement is beyond the scope of our investigation. Studies of GAB1 phosphorylation at Y627, a site involved in the binding and activation of SHP2, revealed constitutive phosphorylation in H3255 cells compared with ligand-induced phosphorylation in H1666 cells (Supplementary Fig. S9), suggesting that differential SHP2 involvement may not result from an inability to complex with GAB1. Still, another marker of defective SHP2 activity [i.e., impaired phosphorylation of SRC at Y418 (35)] was identified in H3255 cells (Supplementary Fig. S9).

We note that we were not able to fully recapitulate the differential phosphorylation of SHP2 and ERK observed in H1666 and H3255 cells in the model systems we explored. Whereas EGF-induced SHP2 Y542 phosphorylation was not detectable in H3255 cells, Shp2 Y542 phosphorylation increased in response to EGF in Egfr<sup>WT/WT</sup> cells infected with retroviruses encoding Egfr<sup>L858R</sup> and HeLa cells expressing Dyn<sup>K44A</sup>. Those increases were more modest, however, than those observed in Egfr<sup>WT/WT</sup> cells infected with retroviruses encoding Egfr<sup>L858R</sup> and HeLa cells expressing Dyn<sup>WT</sup>, respectively. Similarly, relative reductions or delays in ERK phosphorylation observed in these systems with the expression of Egfr<sup>L858R</sup> or Dyn<sup>K44A</sup> were less substantial than the difference observed between H1666 and H3255. However, H1666 and H3255 are not isogenic, so differences in the expression of proteins other than EGFR may affect SHP2 and ERK phosphorylation. Elevated ERBB3 expression in H3255 (and other gefitinib-sensitive NSCLC cell lines), for example, may play a role in modulating SHP2 and ERK phosphorylation dynamics. H1666 cells also carry an uncommon BRAF mutation (G465V), although the implication for altered BRAF or ERK activity in H1666 cells is unclear (36). EGFR expression level can also influence receptor trafficking and signal persistence; we find that EGFR expression in H3255 exceeds that in H1666, in agreement with reports of relative EGFR copy number in these cell lines (30).
Finally, differences in cell background (lung epithelium versus other types) may also affect the extent to which EGFR mutants alter SHP2 and ERK phosphorylation. The failure of MEKDD expression to increase ERK phosphorylation in H3255 cells raises the possibility that additional factors may suppress ERK activity. We explored the possibility that MEKDD expression might increase the activity of MEK phosphatases by probing for MEK phosphorylation at S217/S221. Because both MEKDD isoforms have been mutated at these sites, any reduction in total MEK activity should manifest within the pool of endogenous MEK. We observed no reduction in MEK phosphorylation with MEKDD expression (Supplementary Fig. S10). Interestingly, recent studies showed that transcript levels of MKP3, considered a principal ERK phosphatase, increased more in human bronchial epithelial cells expressing the E746-A750 deletion and L858R EGFR mutants than in those expressing similar levels of wild-type EGFR (37). Thus, the role of MKP3 in determining ERK activity in NSCLC cells with or without EGFR mutation should be explored. Our findings show an important role for ERK in determining cellular response to gefitinib. The observation that MEKDD expression reduced the death response of H3255 cells to gefitinib suggests that even low ERK activity may promote resistance to EGFR kinase inhibition. This is consistent with reports that primary mutations of BRAF and KRAS that increase ERK activity are associated with gefitinib resistance (18). That cotreatment of H1166 cells with gefitinib and U0126 promoted more cell death than treatment with gefitinib alone also suggests a role for ERK activity levels in determining cellular death response to gefitinib. Our finding that prolonged exposure to U0126 before gefitinib administration resulted in further enhanced cell death response may suggest scheduling strategies for the therapeutic combination of MEK and EGFR inhibitors. Our results also raise the notion that the effects of EGFR-activating mutations might be most appropriately characterized as "oncogene imbalance" because the ERK pathway is altered in converse direction to AKT and STAT. This idea resonates with a proposal holding that EGFR mutant expression results in transient prodeath imbalance of survival and apoptotic signaling in response to EGFR inhibition (15). Whereas NSCLC cells expressing EGFR mutants may have abundant PI3K/AKT and STAT activity, they may be put into an especially precarious state by simultaneous impairment of ERK because this pathway can consequently be reduced below critical threshold more easily.

Disclosure of Potential Conflicts of Interest

P.K. Sorg: commercial research grant, Boehringer-Ingelheim; ownership interest, Merrimack Pharmaceuticals; consultant/advisory board, Applied Precision, Vertex, Genentech, and Merrimack Pharmaceuticals. D.A. Lauffenburger: commercial research grant, Boehringer-Ingelheim and AstraZeneca; ownership interest, Merrimack Pharmaceuticals; consultant/advisory board, Merrimack Pharmaceuticals. M.J. Yaffe: ownership interest, Merrimack Pharmaceuticals; consultant/advisory board, Cell Signaling Technology. The other authors declared no potential conflicts of interest.

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