ABSTRACT

Activating mutations in the EGF receptor (EGFR) are associated with clinical responsiveness to EGFR tyrosine kinase inhibitors (TKI), such as erlotinib and gefitinib. However, resistance eventually arises, often due to a second EGFR mutation, most commonly T790M. Through a genome-wide siRNA screen in a human lung cancer cell line and analyses of murine mutant EGFR-driven lung adenocarcinomas, we found that erlotinib resistance was associated with reduced expression of neurofibromin, the RAS GTPase-activating protein encoded by the NF1 gene. Erlotinib failed to fully inhibit RAS–ERK signaling when neurofibromin levels were reduced. Treatment of neurofibromin-deficient lung cancers with a MAP–ERK kinase (MEK) inhibitor restored sensitivity to erlotinib. Low levels of NF1 expression were associated with primary and acquired resistance of lung adenocarcinomas to EGFR TKIs in patients. These findings identify a subgroup of patients with EGFR-mutant lung adenocarcinoma who might benefit from combination therapy with EGFR and MEK inhibitors.

SIGNIFICANCE: The emergence of resistance to EGFR TKIs is a major clinical challenge in the treatment of lung adenocarcinomas driven by mutations in EGFR. This study suggests that, in a subset of patients, resistance is caused by reduced neurofibromin expression, and that in these cases there may be clinical benefit to combining EGFR TKIs with MEK inhibitors.

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See related commentary by Maertens and Cichowski, p. 519.
INTRODUCTION

Lung cancer is the most frequently diagnosed cancer and a leading cause of cancer-related mortality worldwide, accounting for nearly 1.4 million deaths per year (1, 2). Lung adenocarcinoma is the most common histologic subtype of lung cancer, with 10% to 40% displaying activating mutations in the EGF receptor gene (EGFR), occurring most frequently in never-smokers and in East Asian populations (3, 4). The presence of activating EGFR mutations strongly correlates with clinical responsiveness to the EGFR tyrosine kinase inhibitors (TKI) erlotinib and gefitinib. However, although these drugs are initially very effective, resistance eventually arises in almost all patients, resulting in a modest overall survival benefit (3).

Several groups have investigated the mechanisms that underlie resistance to EGFR TKIs. One of the first resistance mechanisms identified in tumors in treated patients was a secondary mutation (T790M) in the EGFR gene (3, 5, 6), which enhances the affinity of EGFR for ATP and reduces binding of the inhibitor (5, 7–9) and accounts for about 50% of resistant cases (5, 6, 10–12).

Additional mechanisms of resistance identified in experimental settings include activation of insulin—like growth factor-1 receptor (IGF-IR), amplification of MET, HER2, or MAPK1, upregulation of the AXL receptor or its ligand, or activating mutations in PIK3CA (13–18). The majority of these genetic alterations have been confirmed in human EGFR-mutant TKI-resistant lung tumor samples, varying in frequency from 5% to 20%. Histologic transformation involved in resistance has also been observed in clinical samples, most prominently the conversion of EGFR inhibitor-sensitive lung adenocarcinomas to drug-resistant small cell lung cancer (SCLC), described in about 5% of cases of acquired resistance to EGFR TKI (10, 12, 19). Less frequently, epithelial-to-mesenchymal transition, potentially related to loss of MED12 or upregulation of AXL, has been reported to result in a broad treatment resistance, including resistance to EGFR TKIs (10, 18, 20, 21). Importantly, the mechanism of acquired resistance is still unknown for about one third of TKI-resistant lung adenocarcinomas (10, 20). In addition, it is evident that multiple mechanisms may contribute to resistance within one tumor (10, 15, 18, 22). Understanding the heterogeneity of molecular mechanisms involved in the evolution of resistance is therefore necessary to optimize the treatment of individual patients with mutant EGFR-driven tumors.

With the aim of improving therapy for EGFR-mutant TKI-resistant lung cancer, we have set out to identify previously unknown mechanisms of resistance to TKIs in this disease. We first performed an in vitro systematic genome-wide analysis to screen for genes whose silencing by siRNA confers resistance to the EGFR inhibitor erlotinib in a human lung cancer cell line that is sensitive to this drug due to the presence of an activating mutation in EGFR. We also took an in vivo approach using a mutant EGFR-driven mouse lung cancer model, analyzing gene expression in tumors associated with the acquisition of resistance to erlotinib. As described previously, these mice develop lung adenocarcinomas that are initially responsive to EGFR TKIs, but develop resistance following repeated cycles of treatment (23). Analysis of the erlotinib-resistant mouse tumors revealed the T790M mutation in about 20% of cases and occasional amplification of Met (23), suggesting that the erlotinib-resistant mouse tumors recapitulate the molecular changes identified in human lung tumors that acquire resistance to EGFR TKIs.

One gene emerging from these two approaches is the negative regulator of RAS proteins, NF1. We show that reduced expression of neurofibromin, the RAS GTPase-activating protein (GAP) product of this gene, is associated with decreased sensitivity of human lung cancer cells to EGFR inhibitory drugs, due presumably to enhanced RAS signaling. Treatment of EGFR-mutant lung cancer cells expressing low levels of neurofibromin with inhibitors of MAP–ERK kinase (MEK), a RAS effector pathway component, restores their sensitivity to EGFR inhibitors. Moreover, the majority of erlotinib-resistant EGFR-mutant mouse lung tumors that do not express the T790M mutation respond to cotreatment with a MEK inhibitor. Finally, we observed reduced NF1 expression in two independent datasets of paired pre- and posttreatment lung adenocarcinomas that acquired resistance to EGFR TKI treatment. We also found that low levels of NF1 expression in pretreatment clinical specimens correlate with poor overall survival in patients with EGFR-mutant lung cancer treated with EGFR TKIs.

Collectively, our data identified low neurofibromin expression as a novel mechanism by which tumor cells are intrinsically less sensitive or acquire resistance to EGFR TKIs and provide a rationale for using drugs targeting MEK in combination with EGFR inhibitors as a therapeutic approach for the treatment of T790M-negative EGFR TKI–resistant lung cancer.

RESULTS

Genome-Wide siRNA Screen Identifies Determinants of Erlotinib Resistance

To identify novel determinants of resistance to the EGFR TKI erlotinib, we performed a genome-wide RNA interference screen examining cell viability in the absence or presence of the drug. We transfected the EGFR-mutant human lung adenocarcinoma–derived PC9 cell line that is exquisitely sensitive to EGFR TKIs with a library of siRNA pools targeting approximately 21,000 unique human transcripts. Forty-eight hours after transfection, culture medium was replaced, and cells were incubated for an additional 72 hours, in the presence or absence of erlotinib (Fig. 1A). The experiment was performed in triplicate for both conditions. We used an erlotinib concentration slightly above the IC_{50} determined for PC9 cells, favoring identification of siRNAs that confer resistance to the drug, but still allowing detection of siRNAs that enhance killing in the same screen.

The effect of the individual siRNA pools on cell survival was analyzed in drug-treated versus untreated conditions (Supplementary Table S1), and we selected siRNAs that showed a substantial differential effect (residual Z score ≥ 2.0 or ≤ −2.0) without killing the untreated cells (control Z score ≥ −2.0; the Z score corresponds to how many SDs away from the mean of the population an individual siRNA lies; see Supplementary Experimental Procedures). Using these criteria, we identified 242 siRNA pools, of which 212 enhanced and 30 decreased cell survival in the presence of erlotinib.
deconvoluted siRNAs could be found (Fig. 1B). Using a cutoff value of ≥ of erlotinib and siRNA-induced knockdown on cell viability sensitivity index that takes into account the individual effects induced silencing on erlotinib sensitivity by determining the gene were analyzed separately.

106 siRNA pools were then taken forward to a deconvolution mentary Fig. S1 and Supplementary Table S2). The validated pools enhanced survival in the presence of erlotinib (Supplementary Table S2). To test the reproducibility of our independent experiment. Ranking the siRNA pools based on scores from this repeat screen revealed that (residual Z score ≤−2.0), whereas siRNAs that killed cells in the absence of the drug (control Z score ≥2.0) are indicated in blue (residual Z score ≤−2.0), whereas siRNAs that killed cells in the absence of the drug (control Z score ≥2.0) are indicated in gray, and siRNAs that have no effect on modulating the response of cells to erlotinib (residual Z score 0.00–0.10) or desensitizing (≤−0.10) effect on cell viability when cultured in the presence of erlotinib.

**Figure 1.** Genome-wide siRNA screen identifies determinants of resistance to erlotinib treatment. A, schematic diagram of the genome-wide siRNA screens performed in P9 cells. Two sets of 384-well plates were reverse transfected with the genome-wide siRNA library, and after 48 h treated with 30 nM/L of erlotinib or left untreated (control) for 72 h. Cells were fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI), and cell number was quantified on a microplate cytomter to obtain a Z score for each siRNA in both conditions. Median Z scores from the triplicate experiments are presented on the graph. siRNAs desensitizing cells to the drug are indicated in red (residual Z score ≥2.0), and sensitizing siRNAs are indicated in blue (residual Z score ≤−2.0), whereas siRNAs that killed cells in the absence of the drug (control Z score ≥2.0) are indicated in gray, and siRNAs that have no effect on modulating the response of cells to erlotinib (−2.0 ≤ residual Z score < 2.0, with control Z score > 2.0) are indicated in black. B, tabulated sensitivity index values of the deconvoluted siRNAs targeting 23 genes, of which two of the four deconvoluted siRNAs had a substantial sensitizing (≥0.10) or desensitizing (≤−0.10) effect on cell viability when cultured in the presence of erlotinib.

( Supplementary Table S2). To test the reproducibility of our findings, we repeated the genome-wide siRNA screen in an independent experiment. Ranking the siRNA pools based on their residual Z scores from this repeat screen revealed that 106 of the previously identified 242 siRNA pools were in the top 5% of the repeat screen list. Almost all of these 106 siRNA pools enhanced survival in the presence of erlotinib (Supplementary Fig. S1 and Supplementary Table S2). The validated 106 siRNA pools were then taken forward to a deconvolution screen, where the four individual siRNA oligos targeting each gene were analyzed separately.

We established the influence of each individual siRNA-induced silencing on erlotinib sensitivity by determining the sensitivity index that takes into account the individual effects of erlotinib and siRNA-induced knockdown on cell viability (24). Using a cutoff value of ≥ 0.10 for sensitizing siRNAs and ≤ −0.10 for desensitizing siRNAs, for 23 siRNA pools a reproducible effect of at least two of four of their individual deconvoluted siRNAs could be found (Fig. 1B).

**Nf1 Downregulation in a Mouse Model of Erlotinib-Resistant EGFR-Mutant Lung Cancer**

One of these genes, Nf1, stood out, as its gene product neurofibromin has a recognized negative regulatory role in signaling downstream of EGFR due to its function as a RAS GAP (25, 26), suggesting a possible mechanistic rationale for its association with acquisition of resistance to EGFR inhibitory drugs. To assess the possible relevance of these *in vivo* results on resistance to EGFR inhibitory drugs *in vivo*, we made use of an inducible mouse model of EGFR-driven lung cancer (23, 27). In this model, expression of mutant EGFR leads to the development of lung adenocarcinomas that are sensitive to erlotinib treatment. Long-term intermittent treatment of these mice with erlotinib, however, leads to the outgrowth of resistant tumors. To assess whether the genes identified in our siRNA screen showed altered expression between untreated (erlotinib-sensitive) tumors and erlotinib-resistant tumors, we compared the expression levels of Nf1 in erlotinib-resistant tumors and corresponding adjacent normal lung using quantitative real-time PCR (qRT-PCR) analysis. These experiments revealed a decrease in Nf1 mRNA levels compared with normal lung in 10 of 18 erlotinib-resistant tumors, of which seven showed a more than 2-fold decrease (Fig. 2A). Comparable results were obtained with two additional qRT-PCR assays using different primer sets (Supplementary Fig. S2). Interestingly, tumors bearing the EGFR<sup>T790M</sup> gatekeeper mutation, Kras mutations, or Met amplification did not show decreased Nf1 expression, suggesting that loss of neurofibromin could be selected for by EGFR TKIs in the absence of other mechanisms of resistance. Gene expression profiling of erlotinib-sensitive and erlotinib-resistant tumors from these mice failed to show significant differential expression of any of the other genes emerging from the siRNA screen of P9 cells listed in Fig. 1B.

Because Nf1 is a known tumor suppressor, neurofibromin expression might be decreased during tumor progression independent of erlotinib treatment. To directly
address this possibility, one would ideally compare erlotinib-sensitive and erlotinib-resistant tumors derived from the same animal. Regrettably, such material is not available. Instead, we analyzed the relative amounts of \(Nf1\) mRNA in EGFR-induced lung tumors and adjacent normal lung from untreated and erlotinib-treated animals. This analysis showed no significant differences in \(Nf1\) expression between normal lung samples and untreated tumors, whereas erlotinib-resistant tumors with an unknown resistance mechanism did express significantly lower levels of \(Nf1\) compared with untreated (erlotinib-sensitive) tumors (Fig. 2B). In an independent set of tumors, we evaluated \(NF1\) protein expression in erlotinib-treated tumors versus untreated tumors and confirmed lower \(NF1\) protein expression in a subset of erlotinib-resistant tumors relative to those that had not been exposed to the drug (Fig. 2C).

Overall, these observations with mouse lung tumors confirm our data with human lung cancer cell lines, showing that low \(NF1\) expression is associated with erlotinib resistance in EGFR-driven lung tumors that lack known resistance mechanisms such as the EGFR(T790M) mutation or Met amplification.
Reduced NF1 Expression Confers Resistance of Lung Cancer Cell Lines to Erlotinib

To validate the possible role of neurofibromin in erlotinib resistance, we introduced into PC9 cells two individual short hairpin RNAs (shRNA; #1 and #2) targeting different non-overlapping regions of the NF1 coding sequence, which are distinct from the previously used siRNA sequences. A nonsilencing scrambled shRNA (shSC) was used as a control throughout the study. Both NF1-targeting shRNAs constructs decreased sensitivity to erlotinib, with a 26-fold increase in the drug concentration required for an absolute survival inhibition of 50% (IC50) for shNF1#1 and a 56-fold increase for shNF1#2 (Fig. 3A) as determined by CellTiter Blue measurement. The shRNA constructs efficiently suppressed neurofibromin mRNA and protein expression, with shNF1#2 having stronger effects. While neurofibromin silencing conferred resistance of PC9 cells to erlotinib or another EGFR kinase inhibitor, gefitinib, the response to chemotherapeutic agents, such as cisplatin or docetaxel, was not affected (Fig. 3B and Supplementary Fig. S3A).

To test longer-term effects of NF1 silencing, we performed colony formation assays in which cells were cultured in the presence of erlotinib for 10 days. NF1 silencing substantially enhanced survival in these assays (Fig. 3C). Prolonged treatment for 4 weeks in a competition assay, in which unlabeled parental PC9 cells were mixed in a ratio of 100:1 with GFP-labeled cells that expressed an shRNA targeting NF1 or a nonsilencing control shRNA, showed substantial outgrowth of the shNF1 cells (Fig. 3D). The percentage of GFP-positive cells remained approximately 1% in the absence of erlotinib, suggesting that NF1 silencing has little or no effect on the basal proliferation of PC9 cells. Selective outgrowth of NF1 knockdown cells was also seen in long-term assays with other lung adenocarcinoma cell lines harboring activating EGFR mutations that are sensitive to erlotinib treatment (Fig. 3E and Supplementary Figs. S3B–S3D). Thus, our data provide evidence to suggest that NF1 silencing reduces the sensitivity of lung adenocarcinoma cells to erlotinib-induced cell death and/or growth arrest.

To exclude off-target effects of the shRNAs, cells were stably transfected with the GAP-related domain (GRD) of neurofibromin or a control empty vector before the shRNA infections. Although NF1 silencing desensitized the control cells to erlotinib treatment, it did not affect erlotinib sensitivity of the neurofibromin GRD-expressing cells, in which the GRD was not targeted by the shNF1 constructs (Supplementary Fig. S3E). In fact, expression of neurofibromin GRD slightly increased erlotinib sensitivity (Fig. 3F). These data confirm a role for neurofibromin in erlotinib response in lung adenocarcinoma cells. Moreover, the observation that the GRD of neurofibromin could restore sensitivity suggests that neurofibromin influences this sensitivity through its function as a negative regulator of RAS proteins, rather than through RAS-independent pathways.

NF1 Silencing Activates the MAPK Pathway in the Presence of Erlotinib

To investigate whether NF1 silencing promotes RAS activation in PC9 cells, we analyzed the amounts of active, GTP-bound RAS in the absence and presence of erlotinib. Although erlotinib reduced the amount of active RAS in both control and NF1-knockdown cells, cells retain substantially higher levels of active RAS in the presence of erlotinib when NF1 expression is reduced (Fig. 4A).

To determine whether this increased RAS activity affects downstream signaling pathways, we examined the phosphorylation status of several downstream signaling proteins (Fig. 4B). Erlotinib reduced the phosphorylation of EGFR and AKT similarly in all cells. Importantly, although erlotinib completely abolished ERK phosphorylation in the parental and control shRNA-infected cells, remaining phosphorylated ERK (pERK) could also still be detected in shNF1 cells (Fig. 4B and Supplementary Fig. S4A) at 1 μmol/L (Supplementary Fig. S4B), which is around the steady-state plasma concentration found in patients treated with erlotinib (28, 29).

Examination of cells expressing the GRD of NF1 revealed that ERK phosphorylation was also completely abolished in these cells in the presence of erlotinib (Fig. 4C). We confirmed an important role for the active MAPK pathway by studying PC9 cells expressing constitutively active forms of MEK (MEK-DD) or AKT [myristylated-AKT (myr-AKT); Supplementary Fig. S4C]. MEK-DD caused a strong decrease in erlotinib sensitivity, whereas the effect of myr-AKT in cell survival assays is more moderate (Fig. 4D and E). The ability of MEK-DD–expressing cells to resist the inhibitory effects of erlotinib can be reversed by treatment with the MEK inhibitor AZD-6244 (Supplementary Fig. S4D). Thus, neurofibromin downregulation increases RAS activity and attenuates the effect of erlotinib on the downstream MAPK pathway, thereby decreasing the sensitivity to EGFR inhibitory drugs.

Cells Expressing Reduced Neurofibromin Respond to Erlotinib in Combination with a MEK Inhibitor

Given the likely importance of the residual ERK phosphorylation for cell survival in the presence of erlotinib, we reasoned that EGFR-mutant cells expressing low neurofibromin levels could be sensitive to treatment with a MEK inhibitor combined with a TKI. Indeed, analyzing a dose–response curve in the presence or absence of erlotinib revealed that the NF1-knockdown cells are resistant to the MEK inhibitor AZD-6244 or erlotinib alone, but do respond to the combination of both inhibitors (Fig. 5A). As expected, the control shSC cells did respond to single-agent erlotinib, as evidenced by the decreased survival at the beginning of the experiment. Similar results were obtained with the MEK inhibitors CI-1040 and PD0325901 (Supplementary Fig. S5A). Although EGFR or MEK inhibition alone is insufficient, these drugs abolished ERK phosphorylation completely in the shNF1 cells when used in combination (Fig. 5B). Similar assays were performed with a clone of PC9 cells that had acquired erlotinib resistance in vivo following prolonged drug exposure that resulted in the emergence of cells with the EGFR gatekeeper mutation T790M (PC9T790M; Supplementary Fig. S5B). These cells could not be resensitized to erlotinib by the addition of a MEK inhibitor (Fig. 5C), and maintain low levels of pERK in the presence of both drugs, in contrast to parental PC9 cells (Fig. 5D).

Although the above studies revealed that MEK inhibition in combination with erlotinib resensitizes erlotinib-resistant shNF1-infected cells in vitro, we continued to examine the sensitizing effect of MEK inhibition to erlotinib in vivo using tumor xenografts. We established xenografts of PC9
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Figure 3. NF1 silencing confers resistance of PC9 lung adenocarcinoma cells to erlotinib (erl). A, silencing of NF1 increases cell survival in the presence of erlotinib. PC9 cells are uninfected (PC9) or stably infected with shRNA containing a nonsilencing control sequence (shSC) or an NF1-targeting sequence (shNF1#1 or #2). Cells were treated with the indicated concentrations of erlotinib for 72 hours before cell viability was measured by CellTiter Blue and normalized to untreated controls. Error bars, SEM. Drug concentrations inducing 50% inhibition in survival (IC50 values; nmol/L) are indicated for each cell line. Knockdown ability of each shRNA is demonstrated at both the mRNA and protein levels using qRT-PCR and immunoblotting, respectively. Error bars, SD. B, silencing of NF1 does not affect the sensitivity to cisplatin. Cells were treated with the indicated concentrations of cisplatin for 72 hours before cell viability was measured and normalized to untreated controls. Error bars, SEM. C, NF1 downregulation enhances cell survival in longer-term colony formation assays. Cells were fixed and stained after 14 days treatment with indicated concentrations of erlotinib. Uninfected PC9 cells or control shRNA-infected cells (shSC) were used as control. D, selective outgrowth of NF1-silenced cells in the presence of erlotinib. GFP-negative PC9 cells were spiked with approximately 1% GFP-positive shRNA-infected cells, nonsilencing control (shSC) or targeting NF1 (shNF1#2), and grown for 4 weeks in the presence or absence of 30 nmol/L erlotinib. Cells were collected and analyzed for GFP expression by fluorescence-activated cell sorting (FACS; X-axis). The percentage of GFP-positive cells is indicated. FSC, forward scatter. E, selective outgrowth of NF1-silenced cells in the presence of erlotinib in NSCLC cell lines HCC4006, HCC827, and H3255. GFP-negative cells were spiked with approximately 1% GFP-positive shRNA-infected cells, nonsilencing control (shSC) or targeting NF1 (shNF1#2), and grown for 1 or 3 months in the presence or absence of 30 nmol/L of erlotinib. Cells were collected and analyzed for GFP expression by FACS (plots presented in Supplementary Fig. S3D). The percentage of GFP-positive cells is indicated. F, expression of NF1-GRD restores sensitivity to erlotinib. Cells were transfected with the NF1-GRD (dotted lines) or empty vector (ev) control (solid lines) before infection with control (shSC) or NF1-targeting shRNAs #1 or #2 (left and right, respectively). Cells were treated with the indicated concentrations of erlotinib for 72 hours before cell viability was measured and normalized to untreated controls. Error bars, SEM. Expression of the NF1-GRD and silencing of endogenous NF1 are shown by qRT-PCR and immunoblot in Supplementary Fig. S3E.
NF1 downregulation activates MAPK pathway signaling. A, NF1 silencing leads to increased levels of RAS activity in untreated and erlotinib-treated conditions. Relative levels of RAS activation were assessed in cells infected with a control shRNA (shSC) or an shRNA targeting NF1 (shNF1#2). RAS immunoblots from RAS pull-down assays are shown (RAS-GTP), along with a RAS immunoblot from total cell lysates as loading control. HRAS, KRAS, and NRAS isoforms are detected in this assay. B, NF1 silencing prevents complete erlotinib-induced ERK dephosphorylation. Parental PC9 cells and cells expressing control shRNA (shSC) or NF1-targeting shRNAs (NF1#1 or NF1#2) were left untreated or treated with 30 nmol/L erlotinib for 1 hour. Cell extracts were immunoblotted to detect the indicated proteins. C, NF1-silenced cells maintain ERK phosphorylation in the presence of erlotinib, which is abolished by reexpression of the NF1-GRD. Cells were transfected with the NF1-GRD (GRD) or empty vector control (ev) before infection with control (shSC) or NF1-targeting shRNAs (shNF1#2). Cells were treated with 30 nmol/L erlotinib for 1 hour, and cell extracts were immunoblotted to detect the indicated proteins. D, active MEK and active AKT desensitizes cells to erlotinib in a short-term survival assay. PC9 cells expressing the ecotropic receptor (Eco) were stably transfected with myr-AKT or MEK-DD and treated with indicated concentrations of erlotinib for 72 hours before cell viability was measured using CellTiter Blue. Cell survival is normalized to untreated controls. Error bars, SEM. E, constitutively active MEK and, to a lesser extent, AKT enhance cell survival in longer-term colony formation assays. Cells were fixed and stained after 14 days of treatment with indicated concentrations of erlotinib.

cells stably infected with a control (shSC) or NF1-targeting (shNF1#2) shRNA. Once tumors were detectable, mice were treated with either erlotinib or AZD-6244, or a combination of both drugs. Combined erlotinib and AZD-6244 treatment for 30 days indeed effectively reduced the tumor growth of both the NF1-silenced and the control tumors. As expected, we did not see a response to erlotinib in the NF1 knockdown tumors, whereas the control tumors did clearly respond (Fig. 5E). In contrast, xenograft tumors of PC9T790M cells failed to respond to the combination treatment (Fig. 5F). These data suggest that modest doses of a MEK inhibitor may resensitize tumors with reduced neurofibromin expression to erlotinib in the absence of the T790M mutation.

Erlotinib-Resistant Mouse Lung Adenocarcinomas Respond to Combined EGFR and MEK Inhibition

Although the above findings suggest a possible treatment opportunity for lung adenocarcinoma cells that express reduced levels of neurofibromin, the effect of combination EGFR and MEK inhibition in established EGFR-driven lung adenocarcinomas that developed resistance to erlotinib remained unknown. We therefore used our tetracycline-inducible mouse model of EGFR-dependent lung cancer, driven by the EGFR<sup>L858R</sup> point mutation, to generate erlotinib-resistant tumors (23). While maintained on a diet containing doxycycline to ensure continued expression of the transgene, the mice were treated with erlotinib for three treatment rounds of 4 weeks each, followed by 4 weeks without drug treatment. Tumors were monitored using micro-CT at the start and end of each treatment period. In a few mice, the tumor response was relatively minor during the second round of erlotinib treatment, and these mice continued on erlotinib (Fig. 6A, M10, 11, 12, 15). As described previously, a diminished response during the third round of erlotinib treatment was commonly observed (23). When possible, we maintained the mice on erlotinib for
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Figure 5. Low neurofibromin-expressing cells respond to erlotinib when combined with MEK inhibition. A, NF1-silenced cells are insensitive to EGFR or MEK inhibitor alone but sensitive to combined drug treatment. Cells expressing control shRNA (shSC) or shRNA targeting NF1 (shNF1#2) were treated with indicated concentrations of AZD-6244 alone (solid line) or in combination with 30 nmol/L of erlotinib (dotted line) for 72 hours before cell viability was measured using CellTiter Blue. Cell survival is shown normalized to untreated controls. Error bars, SEM. B, levels of pERK and phosphorylated AKT (pAKT) are analyzed by Western blotting after treating the shSC- or shNF1#2-infected cells with 30 nmol/L erlotinib or 1 μmol/L MEK inhibitor AZD-6244 (MEKi) alone or in combination for 1 hour. A short and long exposure is shown of pERK. Total ERK and AKT are shown as loading control. C, T790M-positive erlotinib-resistant PC9 (PC9 T790M) cells remain insensitive to erlotinib when treatment is combined with the MEK inhibitor. PC9 T790M and parental PC9 cells are treated with indicated concentrations of MEK inhibitor AZD-6244 alone (straight line) or in combination with 30 nmol/L erlotinib (dotted line) for 72 hours before cell viability was measured and survival normalized to untreated controls. Error bars, SEM. D, levels of pERK and pAKT are analyzed by Western blotting after PC9 and PC9 T790M cells were treated with 30 nmol/L erlotinib or 1 μmol/L AZD-6244 (MEKi) alone or in combination for 1 hour. A short and long exposure is shown of pERK. Total ERK and AKT are shown as loading control. E, NF1-silenced xenograft tumors are sensitive to erlotinib in combination with MEK inhibition, but insensitive to either of both drugs alone. Mice were subcutaneously injected with shSC- or shNF1#2-infected PC9 cells. Once tumors were detectable, mice were randomized to four different treatment regimens: vehicle, erlotinib, AZD-6244 (MEKi), or combined erlotinib and AZD-6244 (erl + MEKi) as described in Supplementary Experimental Procedures. Data, the mean ± SEM tumor volumes for each treatment group over time. P values are indicated for the mice receiving erlotinib alone or combined with AZD-6244 and analyzed against the vehicle control group. n.s., not significant. F, PC9 T790M xenograft tumors are insensitive to erlotinib in combination with MEK inhibition. Mice were injected with PC9 T790M cells. Once tumors were detectable, mice were treated by four different treatment regimens as in E.
Figure 6. Erlotinib-resistant murine EGFR<sup>L858R</sup> lung adenocarcinomas respond to combined EGFR and MEK inhibition. A, line chart depicting the schedule used to treat individual mice with erlotinib alone, MEK inhibitor alone, or both inhibitors combined. Mice were treated 5 days per week for 4 weeks (blue horizontal bars indicate treatment with 25 mg/kg/d of erlotinib), after which treatment was interrupted for 4 weeks (no bars). Erlotinib administration was continued or started sooner in animals that became cachectic during this period (see, for example, mice M4 and M10). The treatment cycle was repeated up to three times, and followed by erlotinib administration alone (blue bars), the MEK inhibitor GSK1120212 alone (gray bars), or combined (red bars, treatment with 25 mg/kg/d erlotinib combined with 2.5 mg/kg/d GSK1120212). B, representative transaxial micro-CT images of lungs of mice before and after the final 4-week treatment round. The left image is from a mouse treated with combined erlotinib and MEK inhibitor, demonstrating a reduction in tumor volume (mouse M11); the middle image is from a mouse treated with erlotinib alone (mouse M6), and the right image is from a mouse treated with MEK inhibitor alone (mouse M13); both showing increased tumor volume. Red arrows point to measurable tumor nodules. C, waterfall plot showing tumor response of the final 4 weeks of combined treatment with EGFR and MEK inhibitors (red bars), the EGFR inhibitor alone (blue bars), or MEK inhibitor alone (gray). Each bar represents one individual tumor, with data expressed relative to the tumor volume measured before the 4-week treatment round, showing progressive disease, stable disease, partial response, or complete response. Tumor mRNA was sequenced for the presence of EGFR<sup>T790M</sup> and analyzed for Met expression; T790M-positive tumors are marked with a T, and tumors showing a >4-fold Met expression are marked with an M. T790M and high Met expression were not detected in the other tumors, or not determined (n.d.) in cases in which no frozen tumor material was available, as indicated in Supplementary Table S3.

Another 4-week round of treatment after the third round, to confirm that the tumors were indeed growing and resistant to long-term erlotinib treatment. Subsequent to the emergence of erlotinib-resistant tumors, mice were treated with erlotinib and the MEK inhibitor GSKit20212, also known as trametinib, for another 4 weeks, or as control with either inhibitor alone (Fig. 6A). We scanned the animals before and after these final 4 weeks of treatment and quantified the tumor volumes (Fig. 6B and Supplementary Fig. S6). As seen in Fig. 6B and C, the combined treatment of EGFR and MEK inhibitors had a striking effect on most erlotinib-resistant mouse lung adenocarcinomas. On the contrary, erlotinib
or MEK inhibitor alone failed to induce significant tumor regression, confirming the resistant nature of these tumors (Fig. 6C). Importantly, combined erlotinib and MEK inhibitor treatment was well tolerated, and mice did not show any sign of weight loss (data not shown).

To assess whether secondary mutations in EGFR were associated with a reduced response to the combined erlotinib and MEK inhibitor treatment, we generated cDNA from RNA that was extracted from individual tumor nodules, and sequenced part of the human EGFR transgene cDNA spanning the transgene (L858R) and T790M region, as described previously (23). We detected the T790M amino acid substitution in two tumors and relatively high Met expression in two other tumors; three of these tumors were treated with combined erlotinib and MEK inhibitor, and these tumors showed a minimal response (Fig. 6C and Supplementary Table S3). As expected, all tumors did express the L858R driver mutation (data not shown). In addition, we evaluated NF1 mRNA expression in these tumors and found that tumors responding well to erlotinib combined with MEK inhibitor more often express relatively lower levels of NF1, but some tumors with higher levels do also respond (Supplementary Table S3), indicating that these tumors might have found alternative mechanisms to activate MEK (16). We furthermore confirmed that all tumors expressed wild-type Kras, Hras, and Nras (codons 12, 13, and 61; data not shown). Collectively, these results demonstrate that erlotinib combined with a licensed MEK inhibitor substantially affects T790M-negative erlotinib-resistant lung adenocarcinomas in mouse models.

Reduced NF1 Expression in Human Lung Adenocarcinoma Samples with Resistance to EGFR Inhibitors

To assess the clinical relevance of this resistance mechanism, we evaluated NF1 expression in 13 human EGFR-mutant lung adenocarcinoma samples that acquired resistance to EGFR TKI treatment compared with matched pretreatment samples (Fig. 7A and B). We were able to extract sufficient RNA from 10 sample pairs to perform NF1 mRNA expression analyses by qRT-PCR (Supplementary Table S4 provides tumor purity and treatment response for each sample presented in Fig. 7A). Four of the erlotinib-resistant samples showed a >2-fold decrease in NF1 expression compared with their matched pretreatment sample, with relatively stable expression in the remaining six pairs (Fig. 7A). Importantly, one of the erlotinib-resistant tumors with reduced NF1 did not harbor an EGFR\textsuperscript{T790M} mutation nor amplified...
MET (sample pair Y10). In addition, using RNA-sequencing (RNA-seq) data from a separate set of three human lung adenocarcinomas treated with EGFR TKIs (erlotinib or gefitinib), we identified reduced NF1 expression in all three posttreatment tumors, with the strongest reduction in the two EGFR TKI–resistant samples that did not express T790M (V1 and V3) and failed to respond to a second-line treatment with afatinib (Fig. 7B). Experiments with the PC9 cell line confirmed that NF1 silencing strongly reduced sensitivity to afatinib as well (Supplementary Fig. S7A). Together, these two clinical datasets indicate that low NF1 might be driving erlotinib resistance in these tumors.

Some posttreatment samples showed reduced NF1 mRNA in the posttreatment tumor co-occurring with an EGFR T790M mutation (sample pairs Y1, Y6, and Y9). Similar observations have been published for other resistance mechanisms (10, 18, 22), which could indicate that multiple mechanisms may contribute to resistance to EGFR TKIs. To address whether heterogeneity in resistance mechanisms could explain our observations, we assessed the abundance of the T790M mutation by pyrosequencing and compared this with the abundance of the original TKI-sensitivity–conferring EGFR driver mutation. In the posttreatment tumor of Y1, 28% of the EGFR present in the posttreatment sample harbored the exon19 deletion, whereas only 7% harbored the T790M mutation. Because a minority of cells in the resistant tumor cells expressed T790M, one can speculate that heterogeneity in resistance might occur in this sample with T790M expression in part of the tumor cells and low NF1 in other cells. A similar heterogeneity is seen in the posttreatment sample of Y9. However, one sample (pair Y6) showed an almost similar abundance of the driver mutation and T790M in the posttreatment sample, 28% and 21%, respectively. We therefore hypothesized that low NF1 and expression of T790M might have additive effects on drug resistance. Indeed, long-term treatment of erlotinib-resistant PC9 T790M cells infected with an shRNA targeting NF1 (shNF1) or a nonsilencing control shRNA (shSC) resulted in selective outgrowth of the shNF1 cells when cultured in the presence of erlotinib or afatinib (Supplementary Fig. S7B). These data suggest that NF1 silencing and T790M have additive effects on the resistance of lung adenocarcinoma cells to EGFR inhibitor-induced death. Combined, the RNA-seq and the qRT-PCR analyses on human samples confirm a clinical relevance of NF1 downregulation in acquired resistance to EGFR TKIs.

To evaluate whether NF1 might be involved in primary resistance as well, we examined NF1 expression in a cohort of 34 NSCLC samples taken at diagnosis from patients who were then treated with erlotinib as first (n = 5) or second (n = 29) line of therapy; the EGFR mutation status of these tumors is unknown. Using the median as cutoff, we found that low NF1 expression was strongly associated with decreased overall survival with a median survival time of 7.6 months (95% confidence interval [CI], 6.6–8.4) compared with 19.1 months (95% CI, 14.0–24.2) for patients with high NF1 expression in their tumor (Fig. 7C). A multivariate Cox regression analysis with NF1 expression, gender, and morphology as input variables confirmed NF1 expression as an independent prognostic factor with a relative risk of 4.1 (95% CI, 1.6–10.7; P = 0.004).

Overall, these clinical data suggest that the level of NF1 expression can determine the responsiveness to EGFR TKI and provide a rationale for testing MEK inhibitors in combination with EGFR TKIs in patients with EGFR-mutant lung cancer.

DISCUSSION

We have used a functional genomic in vitro screening approach, together with a genetically modified mouse lung cancer model system, to investigate mechanisms of acquired resistance to erlotinib in EGFR-mutant lung adenocarcinoma, identifying NF1 as a gene whose loss of function is capable of causing EGFR TKI drug resistance in both settings. Our analyses of two independent sets of paired EGFR-mutant lung adenocarcinoma samples from patients treated with EGFR inhibitors confirm that downregulation of NF1 expression at the time of TKI resistance is a common occurrence in the clinic. Furthermore, NF1 levels may influence the initial response to TKIs, as low neurofibromin in pretreatment specimens is strongly associated with reduced overall survival for patients with EGFR-mutant lung adenocarcinomas treated with EGFR TKI.

Several studies have shown that neurofibromin expression and function can be altered at a number of levels in lung and other cancers. Sequence analysis of the NF1 gene in 188 lung adenocarcinomas revealed mutations in nearly 10% of tumors, most of which lacked coincident KRAS mutations (30). A detailed genomic analysis of human glioblastoma by the Cancer Genome Atlas Research Network showed heterozygous deletion of the NF1 gene resulting in reduced neurofibromin expression, but also low NF1 mRNA without evidence of genomic alterations (31). Our RNA-seq data did not reveal mutations in NF1 in the resistant samples of the 3 patients analyzed (data not shown). We have also sequenced the DNA coding region of NF1 in 10 additional paired samples from patients with mutant EGFR-expressing lung tumors before and after the acquisition of resistance to EGFR inhibitor that do not express EGFR T790M and found no evidence for somatic mutations in these samples either (data not shown). In addition, four of these matched biopsies had sufficient DNA material to analyze the methylation state of the NF1 promoter region, but we found no evidence for differences (data not shown). In our study, 6 out of a total of 13 paired samples show significant downregulation of NF1 mRNA upon TKI resistance. Future studies should evaluate neurofibromin expression at protein level, as additional posttranscriptional regulation could occur as described in glioma cells (32, 33). We have screened multiple antibodies for immunohistochemistry, but none showed the specificity required to detect neurofibromin in human tissue (data not shown).

Some of the human lung cancer samples analyzed in our study showed reduced NF1 expression co-occurring with the T790M mutation, suggesting that multiple mechanisms may contribute to resistance to EGFR TKIs. Such heterogeneity in mechanisms of resistance is consistent with other studies that have described T790M co-occurring with other resistance mechanisms (10, 18, 22). The abundance of transcripts containing the T790M mutation in comparison with those containing only the EGFR driver mutation in these resistant
Role of NF1 in EGFR Inhibitor Resistance

It is expected that novel EGFR inhibitors that specifically inhibit the mutant forms of EGFR while sparing the wild-type protein will reduce such toxicity. Collectively, the present study provides a molecular basis for the combination of EGFR TKI with clinically available MEK inhibitors for the treatment of patients with mutant EGFR-driven lung adenocarcinomas that fail to respond to EGFR TKIs due to either primary or acquired resistance.

METHODS

Cell Culture

H3255, HCC827, and HCC4006 cells were purchased from the American Type Culture Collection Biologics, and PC9 cells were kindly provided by Jeff Settleman (Massachusetts General Hospital Cancer Center and Harvard Medical School). Cells were authenticated by the Cancer Research UK Central Cell Services facility using short-tandem repeat profiling. Cells were cultured in RPMI, supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C and 10% CO2. PC9 and 4T1 cells were generated by continued culturing of PC9 cells with 1 μM/μL erlotinib. Surviving clones were picked and cultured in the presence of 1 μM/μL erlotinib for 3 months, and thereafter without erlotinib.

Genome-Wide siRNA Screen

The genome-wide siRNA library (21,121 siRNAs) was purchased from Dharmacon as the siGENOME SMARTpool collection. siRNA pools targeting PLK1 or UBB1 were used as positive controls for the transfection, and siGENOME RISC-Free Control siRNA, siGENOME Non-Targeting siRNA Pool #2 (scrambled), and siRNA pools targeting Luciferase GL3 were used as negative controls.

Cell Culture

PC9 cells were seeded in 384-well plates (500 cells per well) and reverse transfected with 37.5 nmol/L siRNA using DharmaFECT transfection reagent #2. After 48 hours, the culture medium was replaced by RPMI with 30 nmol/L erlotinib (drug screen) or without erlotinib (control screen). As a control for drug treatment, we left two columns of the control siRNAs untreated in the erlotinib-treated plates. Cells were incubated for 72 hours before being fixed with 80% ethanol, stained with 1 μg/mL of DAPI (Roche) for 1.5 hours, and stored in PBS at 4°C. The number of cells in each well was quantified using an Acumen Explorer microplate cytometer (TTP LabTech). We performed triplicate experiments for both conditions: control and drug screens. We performed an independent genome-wide screen using the same conditions to validate the reproducibility of the hits.

The follow-up deconvolution siRNA screen was performed using the Dharmacon collection siGENOME set of four individual siRNAs targeting a single gene. The deconvolution siRNA screen was performed using the same conditions as in the genome-wide screen.

Details of data analysis of the genome-wide and deconvolution siRNA screens are given in Supplementary Experimental Procedures.

qRT-PCR Paired Human NSCLC Samples

Samples for qRT-PCR analysis were obtained from patients with EGFR-mutant lung adenocarcinoma who developed acquired resistance to erlotinib under Human Investigations Protocol #111000928 (Yale Cancer Center, New Haven, CT). Pre- and posttreatment specimens were reviewed by a pathologist to ensure adequate tumor content. Tumor areas were circled, and manual microdissection was performed to enrich for tumor content in downstream applications. Pyrosequencing was used to determine the abundance of the EGFR L858R and exon 19 DEL mutations as well as the T790M mutation using the EGFR Pyro Kit (Qiagen).
Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue (5 μm × 5 μm slides) using the FFPE RNAeasy Kit (Qiagen). qRT-PCR assays were performed in triplicate with 4.5-ng RNA input per reaction, using the TaqMan RNA-to-Ct 1 Step Kit, and TaqMan primers to amplify NFI and the housekeeping genes ACTB, ESD, and POLR2A, described to be relatively stably expressed in human NSCLC (45). NFI mRNA levels were analyzed using the comparative Ct method and normalized to the average of the three housekeeping genes for individual samples, before the fold change relative to the pretreatment sample was determined.

**RNA-seq Analysis of Human NSCLC Samples**

RNA sequencing was performed as previously described (21), using RNA extracted from FFPE material, with at least 50% tumor content (areas were circled and manual microdissection was performed to enrich for tumor content), from paired pre- and posttreatment biopsies of 3 patients with NSCLC. These patients were treated with either erlotinib or gefitinib followed by afatinib at the Department of Pulmonary Diseases, VU University Medical Center (Amsterdam, the Netherlands). Tumor biopsies were obtained as part of routine medical care after informed consent had been obtained according to the local ethical committee regulations. Tumor EGFR mutational status was determined as previously described (46).

**Overall Survival Analysis of NSCLC Patients Treated with EGFR TKI**

The samples analyzed were retrieved from the diagnostic biobank of the Pathology Service from the Hospital Universitario Marques de Valdecilla (Santander, Spain). For this study, primary tumors were obtained from 34 patients with stage IV non–small cell lung cancer (NSCLC) at the time of diagnosis. None of the patients received chemotherapy or radiotherapy before sampling, and patients received EGFR TKI as primary (n = 5) or secondary (n = 29) treatment upon diagnosis. The presence of an EGFR mutation was not an inclusion criterion, and EGFR mutation status is therefore not available. The study was approved by the local ethics committee.

Total RNA was extracted using the FFPE RNAeasy Kit (Qiagen). qRT-PCR assays were performed in triplicate using the TaqMan RNA-to-Ct 1 Step Kit and TaqMan primers to amplify NF1 and the housekeeping gene ESD. NF1 mRNA levels were normalized to ESD. Patients were divided into two groups, low and high NF1, using the median NF1 expression as the cutoff value. Kaplan–Meier analysis was carried out using SPSS Statistics for the probability of survival (overall survival) in both groups starting from the time of diagnosis.

**Disclosure of Potential Conflicts of Interest**

K. Politi has ownership interest (including patents) in Molecular MD and is a consultant/advisory board member of Takeda. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.C. de Bruin, P.H. Warne


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Role of NF1 in EGFR Inhibitor Resistance

Reduced NF1 Expression Confers Resistance to EGFR Inhibition in Lung Cancer

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