

Preexistence and Clonal Selection of *MET* Amplification in *EGFR* Mutant NSCLC

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SUMMARY

MET amplification activates ERBB3/PI3K/AKT signaling in *EGFR* mutant lung cancers and causes resistance to *EGFR* kinase inhibitors. We demonstrate that *MET* activation by its ligand, HGF, also induces drug resistance, but through GAB1 signaling. Using high-throughput FISH analyses in both cell lines and in patients with lung cancer, we identify subpopulations of cells with *MET* amplification prior to drug exposure. Surprisingly, HGF accelerates the development of *MET* amplification both in vitro and in vivo. *EGFR* kinase inhibitor resistance, due to either *MET* amplification or autocrine HGF production, was cured in vivo by combined *EGFR* and *MET* inhibition. These findings highlight the potential to prospectively identify treatment naive, patients with *EGFR*-mutant lung cancer who will benefit from initial combination therapy.

INTRODUCTION

Epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are effective clinical therapies for patients with advanced non-small cell lung cancer (NSCLC) who have *EGFR*-activating mutations (Asahina et al., 2006; Inoue et al., 2006; Rosell et al., 2009; Sequist et al., 2008; Tamura et al., 2008). A recent phase 3 clinical trial demonstrated that patients with *EGFR*-mutant NSCLC had superior outcomes with gefitinib treatment, compared with standard first-line cytotoxic chemotherapy (Mok et al., 2009). However, despite these dramatic benefits from *EGFR* TKIs in this genetically defined

cohort, all of these patients ultimately develop resistance (referred to here as “acquired resistance”) to gefitinib and erlotinib. Two mechanisms of acquired resistance have been validated in patients. Secondary mutations in *EGFR* itself, including the *EGFR* T790M “gatekeeper” mutation is observed in 50% of resistance cases, and amplification of the *MET* oncogene is observed in 20% of resistance cases (Balak et al., 2006; Bean et al., 2007; Engelman et al., 2007b; Kobayashi et al., 2005; Kosaka et al., 2006; Pao et al., 2005). Both resistance mechanisms lead to maintenance of ERBB3/PI3K/AKT signaling in the presence of gefitinib (reviewed in Engelman and Janne, 2008).

SIGNIFICANCE

The therapeutic success of *EGFR* tyrosine kinase inhibitors (TKIs) in *EGFR*-mutant lung cancers is limited by the development of drug resistance, mediated by *MET* amplification in a subset of patients. Here, we observe that *MET* amplification is present in a small fraction of cells before drug exposure, and its emergence is dramatically accelerated by its ligand, HGF. These findings provide insight into the origins of drug resistance in *EGFR*-mutant cancers and support a rationale for combination treatment strategies as initial therapies, specifically in a molecularly defined cohort of patients with evidence of pre-existing *MET* amplification.

In addition to these genetic alterations, activation of IGF-1R β /IRS-1 signaling through loss of IGF-binding proteins also drives gefitinib resistance in *EGFR* wild-type cancer cell lines (Guix et al., 2008). Additionally, a recent study suggested that the MET ligand, HGF, can promote short-term resistance in two *EGFR* mutated cancer cell lines (Yano et al., 2008). Both ligand-dependent resistance mechanisms maintain PI3K/AKT activation despite EGFR inhibition. However, differences between IGF- and HGF-driven resistance in terms of potency and activation of downstream signaling pathways have yet to be thoroughly examined. Furthermore, the contribution of HGF, if any, to gefitinib resistance mediated by *MET* amplification is unknown.

Strategies for overcoming acquired resistance to gefitinib are now undergoing clinical evaluation. In preclinical studies, the *EGFR* T790M mutation can be overcome by second-generation, irreversible EGFR inhibitors (Engelman et al., 2007a; Kobayashi et al., 2005; Riely, 2008). In addition, the growth of *EGFR*-mutant cancers with *MET* amplification can be inhibited by combined treatment with EGFR and MET kinase inhibitors (Bean et al., 2007; Engelman et al., 2007b). Indeed, there are now clinical trials assessing both irreversible EGFR inhibitors and a combination of MET and EGFR inhibitors in patients with acquired resistance to gefitinib and erlotinib. Furthermore, clinical activity of the irreversible EGFR inhibitor, PF00299804, has been observed in patients with NSCLC who have developed acquired resistance to gefitinib and erlotinib (Janne et al., 2008). As an alternative strategy, to delay or avoid the emergence of resistance, there is increased enthusiasm for using agents that are effective against specific resistance mechanisms as initial systemic therapies. For example, PF00299804 is now being assessed in a phase 2 clinical trial of EGFR TKI-naïve patients. However, there are currently no methods to predict the specific resistance mechanism that a cancer will develop.

In the current study, we modeled in vitro resistance to PF00299804 in the TKI-sensitive *EGFR*-mutant NSCLC cell line HCC827 (Engelman et al., 2006; Engelman et al., 2007b; Ogino et al., 2007). In addition, we evaluated the potency of the MET ligand, HGF, to promote resistance to EGFR TKIs and determined whether *MET* amplification preexists in a subpopulation of cells before treatment with a TKI.

RESULTS

***MET* Amplification Causes Resistance to the Irreversible EGFR Inhibitor PF00299804 by Activating ERBB3 Signaling**

We generated in vitro-resistant clones of HCC827 cells to the irreversible pan-ERBB kinase inhibitor, PF00299804, using methods described elsewhere (Engelman et al., 2006; Engelman et al., 2007b). HCC827 cells were exposed to increasing concentrations of PF00299804, starting with 1 nM, until they were able to proliferate freely in 1 μ M PF00299804, which occurred after six months of drug selection. This concentration was chosen because it is \sim 1000-fold greater than the IC₅₀ for growth inhibition of HCC827 cells and approximately five times greater than the serum concentration of PF00299804 observed in patients with NSCLC in the phase 1 clinical trial (Janne et al., 2008; Schellens et al., 2007). Five independent clones were isolated and expanded for further studies. All five HCC827 PF00299804-

resistant (PFR) clones were resistant to PF00299804 in vitro (Figure 1A and data not shown). No secondary EGFR mutations (e.g., T790M) were detected in any of the clones (data not shown).

We next examined the effects of PF00299804 on EGFR, ERBB3, AKT, and ERK phosphorylation in the HCC827 PFR clones. Unlike in parental HCC827 cells, ERBB3 activation and downstream PI3K/AKT and ERK signaling are maintained in the presence of PF00299804 in HCC827 PFR cells (Figure 1B). We also observed increased total MET protein in the HCC827 PFR cells, and combined MET and EGFR inhibition down-regulated ERBB3, AKT, and ERK phosphorylation as well as the modest EGFR phosphorylation that was maintained in the presence of PF00299804 alone (Figure 1B). This behavior following treatment with PF00299804 alone or in combination with a MET inhibitor is similar to that observed in gefitinib-resistant HCC827 cells (HCC827 GR cells), which were generated in an analogous manner and contained a focal amplification in chromosome 7 harboring the *MET* oncogene (Engelman et al., 2007b).

Given the similarities in the HCC827 PFR and GR cells following treatment with either PF00299804 or gefitinib, respectively, we determined whether the addition of a MET inhibitor would overcome resistance to PF00299804. We used both a tool compound PHA-665,752 and the MET inhibitor PF02341066 currently undergoing clinical development (Figure 1C, upper panel; data not shown) (Zou et al., 2007). The combination of PF00299804 and a MET inhibitor effectively inhibited the growth of HCC827 PFR cells, whereas neither agent alone led to growth inhibition (Figure 1C, upper panel; data not shown). In addition, the combination of gefitinib and PF02341066 also effectively inhibited the growth of HCC827 PFR cells (Figure 1C, lower panel). These findings further suggest that the resistance mechanism in the HCC827 PFR cells is not unique or dependent on the differences between reversible (gefitinib) or irreversible (PF00299804) EGFR inhibitors, but rather is due solely to MET signaling. We also evaluated the effects of the irreversible EGFR inhibitor PF00299804 and the MET inhibitor PF02341066 in an HCC827 PFR xenograft model. Treatment with PF00299804 alone was modestly more effective than treatment with PF02341066 alone, but the tumors demonstrated resistance to PF00299804. However, combined MET and EGFR inhibition completely inhibited tumor growth and produced complete responses ($p < 0.0001$; Figure 1D). In fact, the combination treatment was discontinued after 56 days (Figure 1D, arrow), and no tumor regrowth has been observed to date in any of the xenografts (after more than 35 weeks without therapy) (Figure 1D), suggesting that the mice have been cured.

We next determined whether the increase in MET protein expression was due to *MET* amplification in the HCC827 PFR cells (Figure 2A). All of the PFR clones contained at least a 4-fold amplification of *MET*, similar to the amplification previously observed in the gefitinib-resistant HCC827 (HCC827 GR) cells (Engelman et al., 2007b; Figure 2A). All of the PFR clones also had higher levels of MET protein expression (Figure 2B). Genomewide SNP analysis revealed that the only area of significant copy number gain in HCC827 PFR cells is on distal chromosome 7, similar to that observed in HCC827 GR cells, and contains the *MET* oncogene (Figures 2C and 2D). Furthermore, HCC827 PFR and GR cells share single copy losses of 4p, 5q,

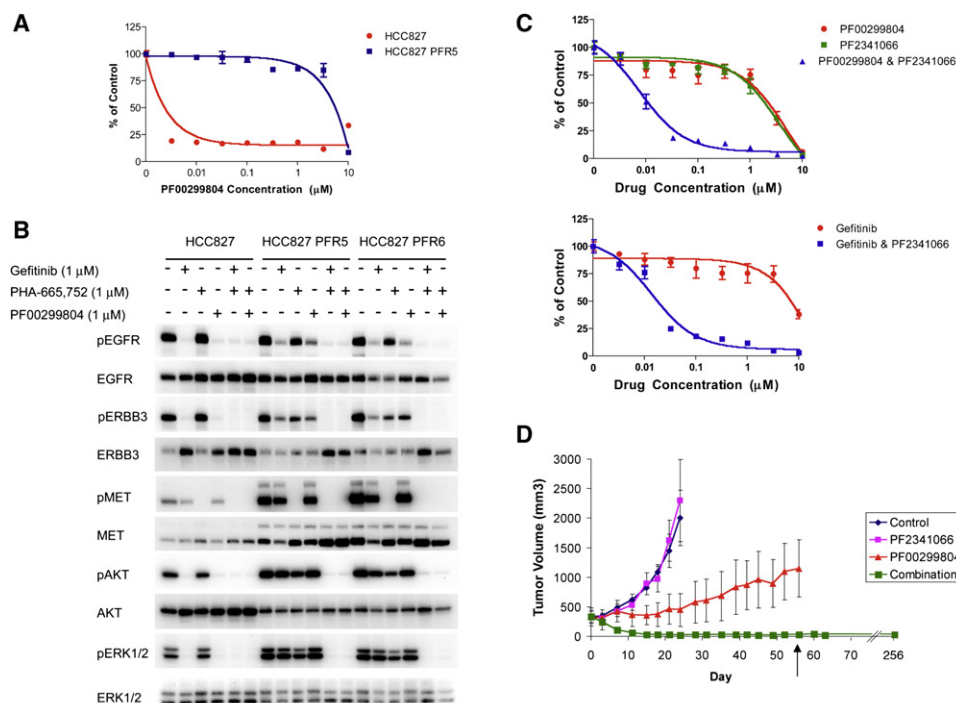


Figure 1. HCC827 PFR Cells Are Resistant to PF00299804, but Combined MET and EGFR Inhibition Blocks PI3K/AKT and ERK Signaling and Restores Sensitivity In Vitro and In Vivo

(A) Parental and resistant HCC827 PFR5 cells treated with increasing concentrations of PF00299804. Cell viability relative to untreated controls measured after 72 hr. Each data point represents the mean \pm SD of 6 wells.

(B) HCC827 and HCC827 PFR5 and PFR6 cells were treated for 6 hr with 1 μ M PF00299804 or gefitinib, PHA-665,752, or their combination. Cell lysates were immunoblotted to detect indicated proteins.

(C) Upper panel: HCC827 PFR6 cells treated with increasing concentrations of PF00299804, PF2341066, or their combination. Lower panel: HCC827 PFR6 cells treated with increasing concentrations of gefitinib alone or in combination with PF2341066. Cell viability relative to untreated controls measured after 72 hr. Each data point represents the mean \pm SD of 6 wells.

(D) HCC827 PFR xenografts in *nu/nu* mice were treated with PF2341066, PF00299804, or their combination. Tumors measured twice weekly. Only combination treatment led to tumor shrinkage and was the most effective treatment in vivo ($p < 0.0001$). Treatment was stopped after 56 days (arrow) and no tumor regrowth was observed in 35 weeks. Each data point represents the mean \pm SD for five mice.

14p, 14q, and 19p, but only HCC827 PFR cells have a single copy loss of 16q. Intriguingly, further examination of the region of *MET* amplification on distal chromosome 7 in both sets of clones showed that, although the copy number changes within the amplicons are not identical in the HCC827 GR and PFR cells, the size and the proximal borders of the amplicons are very similar (Figure 2D). Together these findings, along with the multiple shared regions of single copy genomic loss between the HCC827 PFR and GR cells, suggest that the resistant clones may have arisen from a common origin.

HGF Activates PI3K/AKT Signaling through GAB1 and Leads to gefitinib Resistance

MET amplification was previously shown to cause gefitinib resistance in HCC827 GR cells (Engelman et al., 2007b). We investigated whether activation of MET signaling by its ligand, HGF, could also cause resistance to gefitinib and other ERBB-targeted therapies. In a 72-hr survival assay, HGF induced substantial gefitinib resistance in HCC827 cells that was abolished by the addition of PHA-665,752 (Figure 3A). Furthermore, HGF maintained PI3K/AKT, mTORC1, and ERK activation in the presence of gefitinib in a dose-dependent manner

that mirrored its capacity to maintain cell viability (Figures 3B and 3C).

We also determined the capacity for HGF to maintain downstream signaling and cell viability in other EGFR- and HER2-addicted cancers. In cell lines with *EGFR* exon 19 deletions (HCC827 and PC-9), and an EGFR-driven lung cancer cell line carrying the T790M resistance mutation (H1975), HGF restored PI3K/AKT, mTORC1, and ERK signaling, despite continued EGFR inhibition in the presence of 1 μ M gefitinib or PF00299804 (Figure 3D). HGF also rescued each of these cell lines from TKI-induced cell death after 72 hr (Figure 4A and Figures S1A–S1E, available with this article online). In contrast to the EGFR-addicted cancers, HGF did not rescue *HER2* amplified breast cancer cell lines from the effects of lapatinib (Figure 4A and Figures S1F and S1G), nor did it rescue AKT or mTORC1 signaling in either HER2-driven cell line (Figure 3D). Thus, the capacity to rescue cell viability appears to strongly correlate with capacity to restore downstream signaling, especially along the PI3K/AKT pathway. We suspect that HGF had a minimal effect in BT-474 and SKBR3 cells because these cell lines have lower levels of MET expression than do the other EGFR-driven cell lines that were tested.

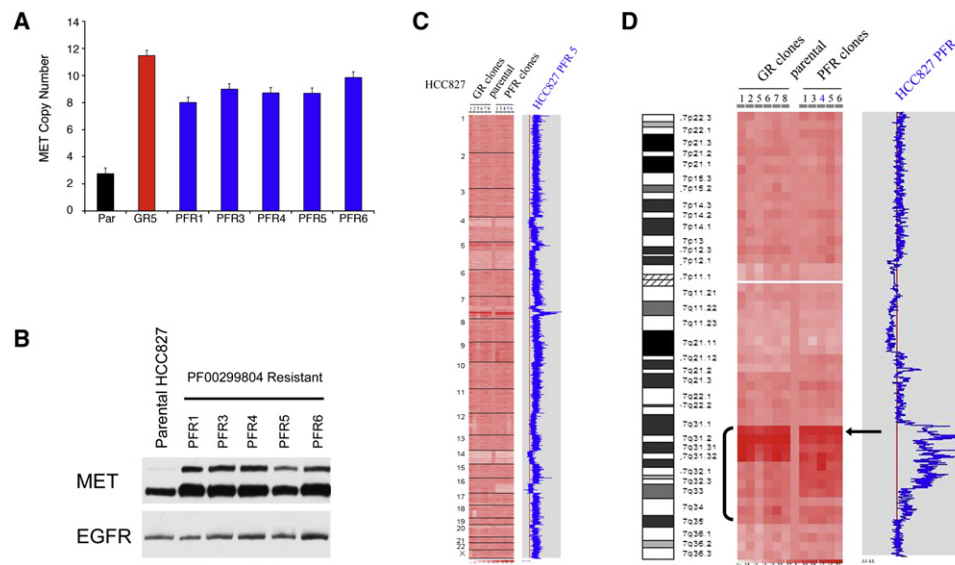


Figure 2. HCC827 PFR Cells Have a Focal Amplification in *MET* That Is Similar to HCC827 GR Cells

(A) *MET* copy number determined by quantitative PCR. Parental (Par) HCC827 and *MET*-amplified HCC827 GR (GR5) cells were used as negative and positive controls, respectively. Each column represents the mean \pm SD for three independent experiments.

(B) Parental HCC827 cells and PFR clones were immunoblotted to detect indicated proteins.

(C) Genomewide view of copy number changes generated using Human Mapping 250K Sty single nucleotide polymorphism (SNP) array and analyzed using the dChip program (see [Experimental Procedures](#)). HCC827 GR clones were compared with HCC827 PFR and HCC827 parental clones. Blue curve indicates degree of amplification of each SNP from 0 (left) to 8 (right).

(D) Chromosome 7 view of copy number changes in HCC827 parental, GR, and PFR cells. Arrow indicates *MET* oncogene.

To confirm the ability of HGF to induce resistance to EGFR TKIs, we introduced the human HGF gene into HCC827 cells (HCC827-HGF). Parental HCC827 cells secrete undetectable levels of HGF; however, HCC827-HGF cells express HGF protein (Figure S2A) and secrete approximately 70 ng/ml HGF into the culture medium (data not shown). Furthermore, HCC827-HGF cells are gefitinib resistant (Figure S2B) and maintain PI3K/AKT, ERK, and mTOR signaling in the presence of gefitinib (Figure S2A); however, gefitinib sensitivity is restored with the addition of a *MET* inhibitor (Figure S2B). We also evaluated the capacity of HGF to induce gefitinib resistance in vivo using an HCC827-HGF xenograft model. We have previously shown that parental HCC827 cells demonstrate complete responses to gefitinib in vivo (Engelman et al., 2006; Engelman et al., 2007a). However, the HCC827-HGF xenografts demonstrated resistance (Figure 3E). Treatment with gefitinib alone was slightly more effective than no treatment or treatment with PF02341066 alone, but only the combination of gefitinib and PF02341066 completely inhibited tumor growth ($p < 0.001$; gefitinib versus gefitinib/PF02341066; Figure 3E). Indeed, three of four mice were cured after 70 days of combined treatment with no evidence of regrowth 70 days after stopping treatment.

Because HGF ligand appeared to be a potent inducer of resistance to RTK inhibitors, we compared its efficacy to that of IGF ligand, which we had previously found to cause gefitinib resistance in A431 cells (Guix et al., 2008). Although IGF exposure led to significant rescue from gefitinib-induced cell death in A431 cells and to partial rescue in HN11 *EGFR* wild-type cells, the other five cell lines tested remained sensitive to ERBB inhibition despite the presence of IGF (Figure 4A and Figure S1). Inter-

estingly, in three of those cell lines (BT-474, HCC827, and H1975), IGF was unable to maintain PI3K/AKT signaling despite potent activation of IGF-1R β (Figure 4B and Table S1). Of note, IGF did not restore ERK phosphorylation in any of the six cell lines examined, including those in which it induced IGF-1R β or PI3K/AKT activation or both (Figure 4B). Thus, unlike IGF, HGF may be more potent at promoting resistance because it leads to activation of both the PI3K/AKT and ERK pathways. Unexpectedly, IGF restored PI3K/AKT signaling in PC-9 cells, but these cells still remained highly sensitive to EGFR inhibition after 72 hr (Figure 4A and Figure S1C). This disconnect between maintenance of PI3K/AKT signaling and lack of an effect on cell viability is not due to a brief, transient restoration of downstream signaling, because we observed that IGF maintained PI3K signaling in PC-9 cells for at least 24 hr in the presence of gefitinib (data not shown).

MET-amplified, gefitinib-resistant HCC827 GR cells utilize ERBB3 as the primary adaptor to activate PI3K/AKT signaling (Engelman et al., 2007b). Although HGF treatment was sufficient to rescue AKT phosphorylation in several EGFR-driven cell lines in the presence of TKIs, ERBB3 phosphorylation was not restored (Figure 3D). This finding suggests that HGF-induced *MET* activation utilizes an adaptor other than ERBB3 to activate PI3K signaling. To determine which PI3K adaptors were being utilized to maintain HGF-mediated PI3K signaling, we immunoprecipitated the p85 regulatory subunit of PI3K and examined coprecipitating phosphotyrosine proteins (Engelman et al., 2005; Engelman et al., 2007b; Guix et al., 2008). As expected, treatment with a TKI disrupted the association of ERBB3 (and other phosphotyrosine proteins) with p85, and the addition of HGF

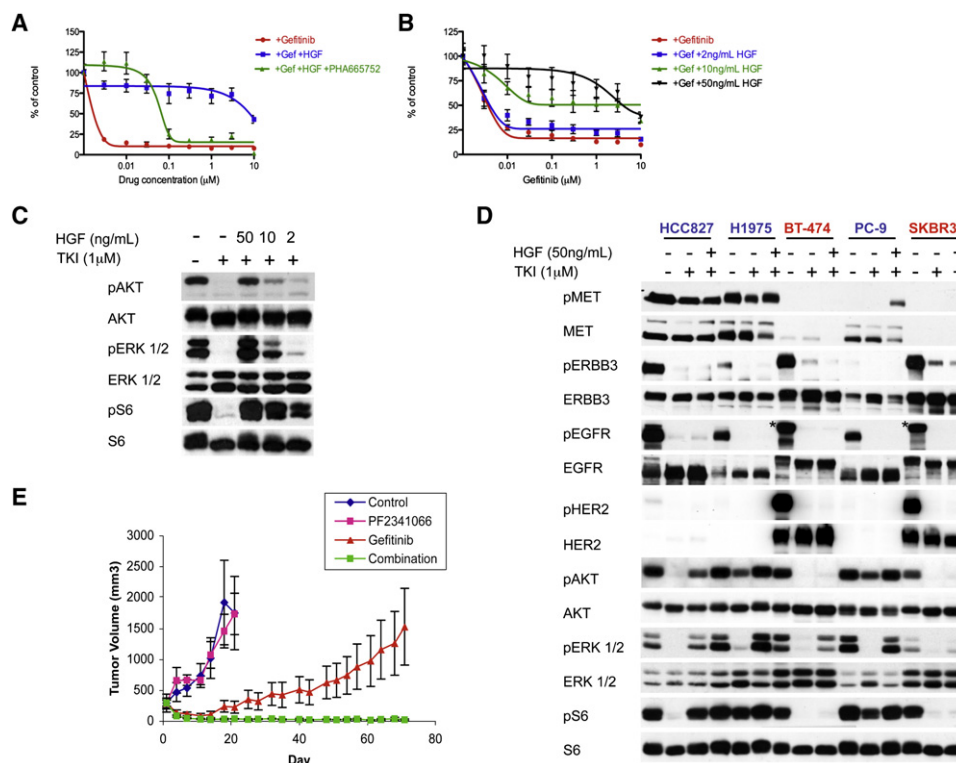


Figure 3. HGF Induces MET-Dependent Resistance Only in Cell Lines in which It Activates PI3K/AKT, ERK, and mTORC1 Signaling

(A and B) HCC827 cells treated with increasing concentrations of gefitinib alone or in combination with PHA-665,752, in the absence or presence of HGF (50 ng/ml) (A), or increasing concentrations of gefitinib alone or in combination with the indicated concentrations of HGF (B). Cell viability relative to untreated controls measured after 72 hr. Each data point represents the mean \pm SD of 6 wells.

(C) HCC827 cells were treated for 6 hr with 1 μ M gefitinib alone or in combination with the indicated concentrations of HGF. Cell lysates were immunoblotted to detect indicated proteins.

(D) Cells were treated for 6 hr with gefitinib (HCC827, PC-9), PF00299804 (H1975), or lapatinib (BT-474, SKBR3), alone or in combination with HGF (50 ng/ml). All drugs were used at 1 μ M. Cell lysates were immunoblotted to detect indicated proteins. Asterisk indicates cross-reaction by the p-EGFR antibody against p-HER2. Cell lines in which HGF rescued viability are labeled in blue, and cell lines in which HGF did not rescue viability are labeled in red.

(E) HCC827-HGF xenografts in *nu/nu* mice treated with PF2341066, gefitinib, or their combination and tumors measured twice weekly. Some growth inhibition was observed with gefitinib alone; however, only combination treatment led to complete tumor shrinkage ($p = 0.002$). Each data point represents the mean \pm SD for five mice. See also Figure S2.

did not restore the interaction (Figure 5A). However, we observed that HGF potently induced the association between p85 and Grb2 associated binder 1 (GAB1), which runs as a broad, highly tyrosine-phosphorylated band at approximately 110 kDa.

To more directly assess whether GAB1 mediates HGF-mediated activation of PI3K/AKT signaling and cell viability, we used small interfering RNA (siRNA) to knockdown GAB1 expression in the HCC827 cells. Knockdown of GAB1 reduced HGF-mediated rescue of PI3K/AKT signaling (Figure 5B) and inhibited the ability of HGF to rescue HCC827 cells from gefitinib-induced cell death (Figure 5C). Of note, although the addition of HGF leads to substantial loss of GAB1 protein (Figure 5B), the amount of tyrosine phosphorylated GAB1 is dramatically increased (Figure S3), and this facilitates the efficient coupling to PI3K (Figure 5A). Thus, activation of HGF/MET signaling can lead to gefitinib resistance in *EGFR*-mutant cancers by activating PI3K/AKT signaling through two different adaptors: ERBB3 when MET is activated by genomic amplification or GAB1 when MET is activated by HGF.

Transient HGF Exposure Leads to Stable Ligand-Independent Gefitinib Resistance in HCC827-50GR Cells through Selection of a Preexisting *MET*-Amplified Clone

Because HGF-induced resistance to EGFR TKIs appears intimately linked to ligand-induced activation of downstream signaling, we hypothesized that long-term resistance would require continuous exposure to HGF. We observed that, by replenishing cells with HGF in combination with the EGFR TKI every 3 days, cells continue to be highly resistant indefinitely (data not shown). Thus, we treated each cell line with HGF in the presence of an EGFR inhibitor for 14 days and then removed HGF, but maintained the cells in the EGFR TKI. Surprisingly, HCC827 cells treated transiently with HGF remained permanently resistant to gefitinib after HGF withdrawal (Figures 6A and 6B). These stably resistant cells were termed HCC827-50GR (50 ng HGF Gefitinib Resistant) cells (Figure 6A). In contrast, HCC827 cells that are not pretreated with HGF develop gefitinib resistance only after six months of gradually increasing

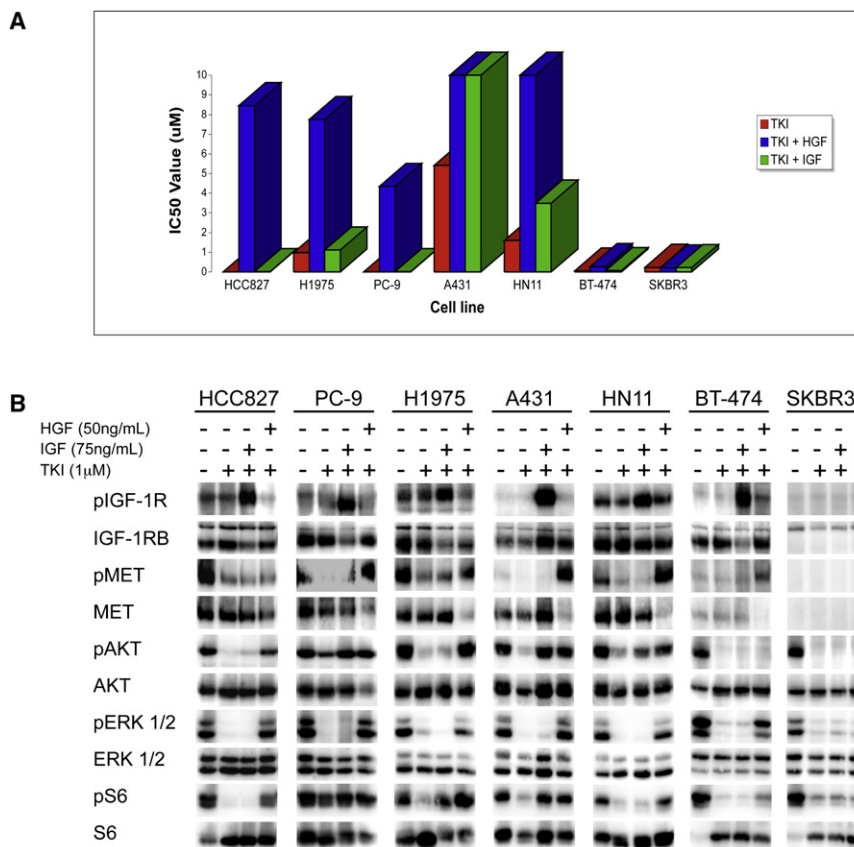


Figure 4. IGF Rescues PI3K/AKT and mTORC1 Signaling in Some Cell Lines but Fails to Activate ERK

(A) IC₅₀ values for viability curves (Figure S1) in the presence or absence of HGF and IGF. Cells were treated with increasing concentrations of the appropriate TKI alone (red) or in combination with 50 ng/ml HGF (blue) or 75 ng/ml IGF (green). (B) Cells were treated for 6 hr with gefitinib (HCC827, PC-9, A431, HN11), PF00299804 (H1975), or lapatinib (BT-474, SKBR3) alone or in combination with HGF (50 ng/ml) or IGF (75 ng/ml). All drugs were used at 1 μM. Cell lysates were immunoblotted to detect indicated proteins. BT-474 and SKBR3 cell lysates were run on the same gel, and no MET or IGF-1Rβ was detected in SKBR3 cells relative to BT-474 cells. See also Table S1 for quantification.

concentrations of drug exposure (Engelman et al., 2007b). In addition, when HCC827-50GR cells were grown in media alone (without gefitinib) for eight weeks, these cells (HCC827-50GR [8wksR5]) maintained their resistance (Figure S4A). Treatment with HGF alone (without gefitinib) for 14 days did not yield stably resistant cells (Figure S4C and Table S2). Thus, lasting resistance conferred by transient HGF requires the selective pressure of gefitinib during ligand exposure.

Stably resistant HCC827-50GR cells maintained PI3K/AKT, mTORC1, and ERK activation in the presence of gefitinib. Surprisingly, ERBB3 also remained phosphorylated in HCC827-50GR cells treated with gefitinib (Figure S4B), which suggests that, although initial HGF-mediated resistance mechanisms utilized GAB1 to activate PI3K/AKT signaling, the ligand-independent HCC827-50GR cells utilize ERBB3 to activate PI3K/AKT signaling. This observation suggests that short-term exposure to HGF may lead HCC827 cells to develop or select the same mechanism of stable resistance, through activation of ERBB3/PI3K signaling, as was observed in *MET*-amplified HCC827 GR cells (Engelman et al., 2007b). Unlike the HCC827 cells, several other EGFR-driven cancer cell lines that were made resistant to EGFR TKIs by HGF treatment did not maintain stable ligand-independent resistance after the withdrawal of HGF (Figures S4D–S4F and Table S3). These findings suggest that HCC827 cells are uniquely poised to develop stable ligand-independent resistance.

Stably resistant HCC827-50GR cells had increased total MET protein levels, compared with parental cells, and maintained

amplification may be driving ERBB3/PI3K/AKT signaling and gefitinib resistance in HCC827-50GR cells.

To examine this hypothesis, we exposed HCC827-50GR cells to PHA-665,752 alone or in combination with gefitinib. Only the combination of gefitinib and PHA-665,752 resulted in a substantial reduction in the number of viable cells (Figure 6D, upper panel). In addition, the HCC827-50GR (8wks R5) cells (grown in media without gefitinib for eight weeks) also remained sensitive only to the combination of MET and EGFR inhibition (Figure 6D, lower panel). Furthermore, treatment with gefitinib in combination with PHA-665,752 completely blocked ERBB3 phosphorylation as well as downstream PI3K/AKT, mTORC1, and ERK signaling in HCC827-50GR and HCC827-50GR(8wks R5) cells (Figure 6E). Taken together, these results suggest that MET inhibition restores EGFR dependence and gefitinib sensitivity in HCC827-50GR cells.

These results led us to examine tissue sections from HCC827-HGF xenograft models treated with gefitinib (Figure 3E). Of three tumors that developed gefitinib resistance, one exhibited significant *MET* amplification (Figure 7A). Thus, *MET* amplification is also facilitated by HGF in vivo.

Because HCC827 GR, PFR and 50GR cells all eventually develop focal *MET* amplification as a resistance mechanism, we hypothesized that parental HCC827 cells may harbor a preexisting *MET*-amplified clone. We analyzed 4237 individual HCC827 cell nuclei using high-throughput FISH (Experimental Procedures) and identified six (0.14%) of 4237 cells that harbored significant *MET* copy number gains (Figures 7B and

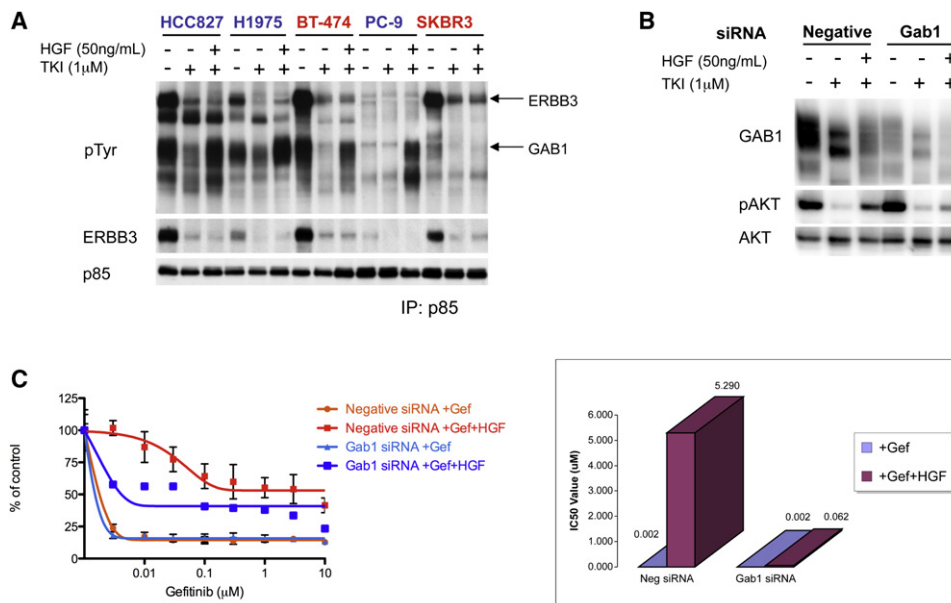


Figure 5. HGF Rescue of PI3K/AKT Signaling Is Mediated through GAB1 Instead of ERBB3

(A) Cells treated for 6 hr with gefitinib (HCC827, PC-9), PF00299804 (H1975), or lapatinib (BT-474, SKBR3), alone or in combination with HGF (50 ng/ml). All drugs were used at 1 μ M. Cell extracts were immunoprecipitated with an anti-p85 antibody followed by western blot with anti-p-Tyr, anti-ERBB3, and anti-p85 antibodies.

(B) HCC827 cells were transfected with a negative control or GAB1 siRNA for 48 hr. Transfected cells were treated for 6 hr with gefitinib (1 μ M) alone or in combination with HGF (50 ng/ml). Cell lysates were immunoblotted to detect indicated proteins. See also Figure S3.

(C) HCC827 cells were transfected with GAB1 siRNA or a negative control siRNA for 48 hr, then treated with increasing concentrations of gefitinib, alone or in combination with 50 ng/ml HGF. Left panel: cell viability relative to untreated controls measured after 72 hr. Each data point represents the mean \pm SD of 6 wells. Right panel: plot of IC₅₀ values corresponding to cell viability curves.

7C). These results were confirmed in an independent experiment using a second gefitinib-sensitive parental HCC827 cell line (HCC827 N1; Figure 7C). We also generated two subclones derived from single cells from the gefitinib-sensitive parental HCC827 cell line (HCC827 C1 and C2). Both subclones were sensitive to gefitinib in vitro (data not shown), and each also contained a low-frequency population of *MET*-amplified cells (Figure 7C). We further examined the gefitinib-sensitive H3255 and PC-9 cells using FISH. Gefitinib-resistant clones of both H3255 and PC-9 have been isolated and reported to contain the *EGFR* secondary resistance mutation T790M but not *MET* amplification (Engelman et al., 2006; Ogino et al., 2007). We did not detect a subpopulation of *MET*-amplified cells in the H3255 or the PC-9 cells (Figure 7C).

We hypothesized that the mechanism by which transient treatment with HGF and gefitinib leads to the generation of *MET*-amplified HCC827-50GR cells is by selecting this small population of preexisting *MET* amplified cells out from the parental HCC827 cell population. To test this hypothesis, we spiked unlabeled HCC827 parental cells with 0.1% of either GFP-labeled HCC827 cells or GFP-labeled *MET*-amplified HCC827 GR6 cells. We treated these two populations with either media alone (no selection) or with gefitinib in combination with HGF. Media was changed and fresh HGF was added every 72 hr, and cells were collected after 19 days for FACS to quantify the percent of cells with GFP expression (Figure S5A). As expected, there was no significant change in the percentage of GFP-labeled HCC827 cells at the end of 19 days. However,

the percentage of GFP labeled *MET*-amplified HCC827 GR6 cells increased over 300-fold to almost 33% in just over two weeks (Figure 7D). Taken together, these results suggest that HGF exposure in the presence of an EGFR inhibitor leads to the rapid selection of a preexisting *MET*-amplified clone in the HCC827 cells (Figure S5B).

Analyses of Tumors with Acquired Resistance to Gefitinib/Erlotinib Reveal Evidence of Pretreatment *MET* Amplification and Increased HGF Expression in Resistant Cancers

To determine the clinical implications of these in vitro and in vivo observations, we examined tumor specimens from gefitinib- or erlotinib-treated patients with *EGFR*-mutant NSCLC (Figure 8). All patients had a clinical partial tumor response to gefitinib or erlotinib treatment and subsequently developed clinical drug resistance. We evaluated 27 patients, 16 with paired specimens obtained before and after gefitinib or erlotinib treatment and 11 with drug resistance specimens alone. All specimens, when feasible, were evaluated for *MET* amplification, HGF expression by immunohistochemistry (IHC), and the presence of *EGFR* T790M (Figure 8 and Figure S6). We observed *EGFR* T790M in 55% (15 of 27) and *MET* amplification in 15% (4 of 27) of resistant tumor specimens. In patients with paired tumor specimens, HGF expression was higher in the drug-resistant specimens than in the pretreatment specimens ($p = 0.025$, Wilcoxon signed-rank test). In patients with drug-resistant specimens alone, HGF expression was similar to that of drug-resistant specimens in

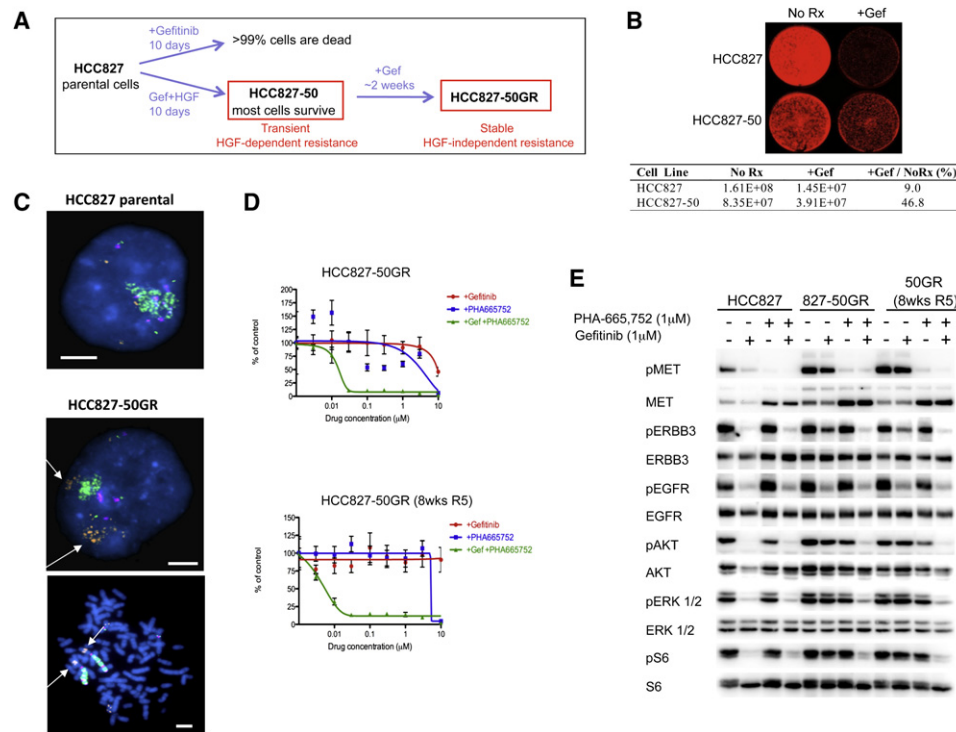


Figure 6. Transient HGF Exposure Leads to *MET* Amplification and Stable Ligand-Independent Gefitinib Resistance in HCC827 Cells

(A) HCC827 cells treated with HGF (50 ng/ml) and 1 μ M gefitinib are resistant to gefitinib (HCC827-50 cells). After the removal of HGF, stably resistant HGF-independent HCC827-50GR cells survive in 1 μ M gefitinib alone. In contrast, parental HCC827 cells do not survive when treated with 1 μ M gefitinib. (B) Parental HCC827 cells and HCC827-50 cells (pretreated with gefitinib in combination with HGF [50 ng/ml] for 14 days) were grown in media alone (No Rx) or media treated with 1 μ M gefitinib (+Gef) for 7 days. Viable cells were visualized and quantified using Syto60 staining. (C) Fluorescence in situ hybridization (FISH) of *MET/EGFR/CEP7* probe set with HCC827 and HCC827-50GR cells: *MET*, orange; *EGFR*, green; and *CEP7*, aqua. Metaphase spread (bottom) shows multiple copies of *EGFR* and *MET* (arrow) on individual chromosomes. Scale bars represent 10 μ m. (D) HCC827-50GR cells (upper panel) and HCC827-50GR cells grown in media alone (without gefitinib) for 8 weeks, 50GR 8wks R5 (lower panel), were treated with increasing concentrations of gefitinib or PHA-665,752 or their combination for 72 hr. Cell viability was measured relative to untreated controls. Each data point represents the mean \pm SD of 6 wells. (E) HCC827 cells and stably resistant HCC827-50GR cells were treated for 6 hr with gefitinib, PHA-665,752, or their combination. All drugs were used at 1 μ M. Cell lysates were immunoblotted to detect indicated proteins. See also Figure S4 and Tables S2 and S3.

patients with paired tumor specimens. Together, these findings support our in vitro and in vivo studies on HGF mediating resistance to EGFR TKIs.

We further evaluated the pretreatment specimens for evidence of *MET* amplification. In all four patients with *MET* amplification in the drug-resistant specimens, we observed rare (<1%) tumor cells with *MET* amplification from the corresponding pretreatment specimens (Figures 8A and 8B). In contrast, of eight cases of resistant cancers without *MET* amplification, we observed rare *MET*-amplified tumor cells in only one corresponding pretreatment tumor specimen. These findings are consistent with cell line data (Figures 7B and 7C), where we observed evidence of preexisting *MET* amplification only in the cell line that subsequently develops *MET* amplification as its resistance mechanism.

DISCUSSION

Kinase inhibitors have emerged as effective clinical therapies for cancers that exhibit oncogene addiction to a particular kinase. (Demetri et al., 2002; Druker et al., 2001; Inoue et al., 2006;

Mok et al., 2009; Sequist et al., 2008). However, the clinical success of treatment with kinase inhibitors is uniformly limited by the development of drug resistance. To date, resistance mechanisms have predominately involved secondary genomic alterations in the target kinase that alter either the physical (such as steric hindrance) or biochemical (change in ATP affinity) properties of the receptor and result in drug resistance (Gorre et al., 2001; Shah et al., 2002; Yun et al., 2008). We have previously described *MET* amplification as a mechanism of gefitinib resistance in *EGFR*-mutant cancers (Engelman et al., 2007b), leading to persistent activation of both PI3K/AKT and ERK signaling in the presence of the EGFR TKI (Engelman et al., 2007b).

A critical question for all resistance mechanisms to kinase inhibitors is whether they occur as a result of treatment or whether they preexist prior to treatment and are selected out during the course of therapy. At least some imatinib-resistant CML clones are thought to be present at low levels before treatment and undergo clonal selection during imatinib exposure (Hofmann et al., 2003; Roche-Lestienne et al., 2002, 2003; Shah et al., 2002). Similarly, *EGFR* T790M can be detected at

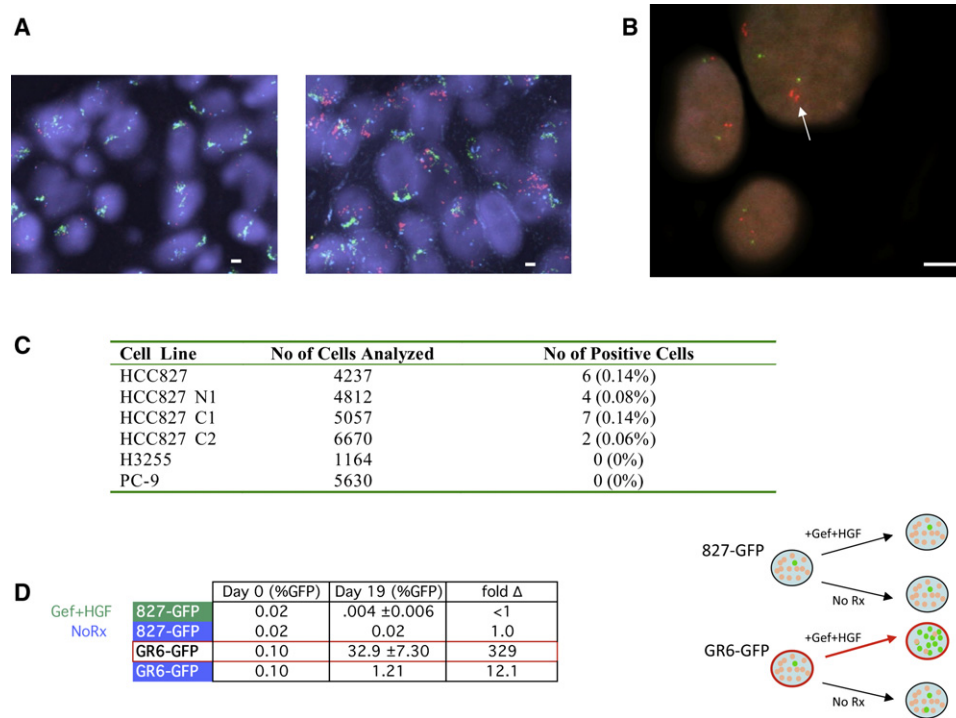


Figure 7. HGF Treatment Selects out a Small Preexisting Population of *MET*-Amplified HCC827 Cells from the Parental Population In Vitro and In Vivo

(A) Fluorescence in situ hybridization (FISH) of *MET/EGFR/CEP7* probe set: *MET*, red; *EGFR*, green; and *CEP7*, aqua. Left panel, tumor sections from control HCC827 xenograft models that do not express HGF showed normal *MET* copy number. Right panel, tumor sections from one of three HCC827-HGF xenografts treated with gefitinib (Figure 3E) showed significant *MET* amplification.

(B) High-throughput FISH analysis of HCC827 cells identifies a subpopulation harboring *MET* amplification (arrow). *MET* (RP-11-951120; red); 7qter (RP-11-6903; green). All scale bars represent 10 μ m.

(C) Parental HCC827 cells and three independent clones harbor a small percentage of *MET*-amplified cells. No preexisting *MET* amplification was detected in H3255 or PC-9 cell populations.

(D) Left panel: HCC827 cells were spiked with approximately 0.1% of GFP-labeled HCC827 cells or GFP-labeled *MET* amplified HCC827 GR6 cells. Each population was grown in either media alone or media treated with gefitinib (1 μ M) with HGF (50 ng/ml). Cells were collected after 19 days, and GFP levels were quantified using FACS. Each data point for cells treated with gefitinib plus HGF represents the mean \pm SD for 3 independent wells. Fold change is the ratio of Day 19 to Day 0 (%GFP). Right panel: diagrammatic depiction of results. See also Figure S5.

low levels in patients with *EGFR*-mutant NSCLC prior to gefitinib or erlotinib treatment (Maheswaran et al., 2008). Our current findings provide support that this may also be the case for *MET* amplification both in HCC827 cells (Figure S5B) and in patients with *EGFR* mutant NSCLC who subsequently develop *MET* amplification at the time of clinical gefitinib or erlotinib resistance (Figure 8). The identification of a drug resistance mechanism from a pretreatment tumor specimen provides the opportunity to specifically target that resistance mechanism before its emergence. This approach is clinically appealing because combined treatment with an *EGFR* and *MET* inhibitor, specifically in patients with evidence of *MET* amplification at baseline, may lead to a longer time to progression than is currently observed with gefitinib or erlotinib alone (Asahina et al., 2006; Inoue et al., 2006; Mok et al., 2009; Rosell et al., 2009; Sequist et al., 2008; Tamura et al., 2008). In fact, combined *EGFR* and *MET* inhibition in HCC827 cells extinguishes the emergence of *MET*-amplified drug-resistant clones (data not shown). However, it will be critical to learn whether upfront treatment with combination therapy is tolerable (toxicity) and will provide more clinical benefit than treatment at the time of relapse.

Intriguingly, HCC827 cells appear to be predisposed to the development of low-level *MET* amplification, because subclones of cells expanded from single-cell clones derived from parental HCC827 cells (HCC827 N1 and N2) also are found to contain low levels of *MET* amplification (Figure 7C). *MET* is located at a fragile site in chromosome 7, which facilitates its amplification, and subsequently a selection for clones harboring *MET* amplification can occur under drug pressure (Hellman et al., 2002). Why this occurs only in the HCC827 cells and a subset of lung cancers, and not in other *EGFR*-mutant cell lines and cancers, is currently unknown. Collectively, these studies suggest, but do not prove that the specific mechanisms of resistance that will develop as a result of drug exposure may be predetermined and occur as a result of drug selection. Understanding why some *EGFR*-mutant cancers are predisposed to develop *MET* amplification will help further refine the clinical development of *EGFR* and *MET* inhibitor combinations.

In this study, we also demonstrate two different and distinct roles for HGF in mediating *EGFR* TKI resistance. First, HGF can independently rescue both PI3K/AKT and ERK signaling in the presence of gefitinib and lead to drug resistance both

A

Paired Specimens							
Pre-Treatment				Drug Resistant			
#	EGFR mutation	MET Amp	HGF Score	EGFR mutation	T790M	MET Amp	HGF Score
1	Exon 19 del	No	30	Exon 19 del	No	No	200
2	Exon 19 del	No	50	Exon 19 del	No	No	120
3	Exon 19 del	No	N/A	Exon 19 del	Yes	No	300
4	Exon 19 del	Yes (< 1%)	200	Exon 19 del	No	Yes	300
5	Exon 19 del	Yes (< 1%)	120	Exon 19 del	Yes	No	200
6	Exon 19 del	No	200	Exon 19 del	No	No	200
7	Exon 19 del	N/A	400	Exon 19 del	Yes	No	350
8	L858R	N/A	N/A	Exon 19 del	Yes	No	350
9	G719S, S768I	No	95	L858R	Yes	No	90
10	L858R	Yes (< 1%)	60	None*	N/A	No	60
11	Exon 19 deletion	N/A	70	L858R	No	Yes	400
12	L858R	N/A	100	Exon 19 del	Yes	No	300
13	Exon 19 del	No	300	L858R	No	No	50
14	L858R	No	40	Exon 19 del	Yes	No	145
15	Exon 19 del	Yes (< 1%)	0	L858R	No	No	180
16	Exon 19 del	Yes (< 1%)	100	Exon 19 del	Yes	Yes	100
				Exon 19 del	Yes	Yes**	150
Resistant Only							
17				Exon 19 del	Yes	No	180
18				Exon 19 del	Yes	No	200
19				L858R	Yes	No	200
20				Exon 19 del	Yes	No	300
21				Exon 19 del	Yes	No	200
22				Exon 19 del	Yes	No	80
23				Exon 19 del	No	No	30
24				L858R	No	No	400
25				Exon 19 del	No	No	N/A
26				Exon 19 del	No	No	N/A
27				Exon 19 del	Yes	No	0

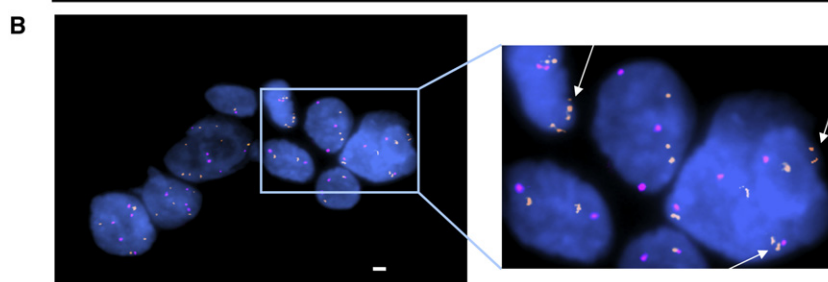


Figure 8. HGF Expression and Preexisting *MET* Amplification Can Be Detected in Tumor Specimens from Patients with NSCLC

(A) Summary of tumors from gefitinib- or erlotinib-treated patients, including 16 paired and 11 drug-resistant samples only. Samples were evaluated for *EGFR* mutational status, *MET* amplification, and HGF expression. Single asterisks indicate that specimen contained <30% tumor cells. Double asterisks denote *MET* amplification defined by qPCR, as described elsewhere (Engelman et al., 2007b). Data on *EGFR* T790M and *MET* amplification in resistant specimens only from patients 1–4 and 17–19 have been previously published (Engelman et al., 2007b). N/A, not available. (B) FISH analysis of pretreatment sample from patient 10 shows evidence of a subset of *MET*-amplified cells (arrow) before exposure to an EGFR TKI: *MET*, RP-11-951120, orange; *CEP* 7, aqua. Scale bars represent 10 μ m. See also Figure S6.

erative advantage to a subset of cells with high *MET* expression (those with amplification), thus facilitating their rapid clonal expansion. Activation of *MET* signaling is a unique resistance mechanism to kinase inhibitors because it can occur through multiple independent mechanisms, amplification or ligand mediated, and when combined can lead to rapid evolution of drug resistance.

Our current findings provide insight into future therapeutic strategies for the treatment of *EGFR*-mutant NSCLC.

in vitro and in vivo. Unlike in *MET*-amplified resistant cancers, HGF-mediated resistance occurs through GAB1, not ERBB3, signaling. Higher levels of HGF can be detected in tumor specimens from patients with NSCLC that are clinically resistant to gefitinib or erlotinib, compared with pretreatment tumor specimens (Figure 8A). Notably, in some patients without evidence of *EGFR* T790M or *MET* amplification, HGF expression is greater in the resistant specimen (patients 1 and 14; Figure S6C) than in the pretreatment specimen, supporting a role for HGF alone in promoting drug resistance. This finding is consistent with prior observations (Yano et al., 2008). Ligand-mediated drug resistance is unique to HGF because IGF does not rescue TKI-induced cell death in the majority of cell lines tested. Surprisingly, IGF did not restore PI3K/AKT signaling in most *EGFR*-mutant cancers, despite substantial levels of IGF-1R β expression and tyrosine phosphorylation. Furthermore, unlike HGF, IGF did not restore ERK signaling even in cell lines in which it restored PI3K/AKT signaling in the presence of a TKI. These signaling differences between HGF and IGF may underlie the lack of drug resistance induced by IGF. In its second role, HGF accelerates the emergence of *MET* amplification in HCC827 cells both in vitro and in vivo. Intriguingly, this process requires concomitant EGFR inhibition, because HGF exposure alone does not lead to emergence of *MET*-amplified clones. It is possible that, in the presence of EGFR inhibition, HGF provides a unique proliferative

advantage to a subset of cells with high *MET* expression (those with amplification), thus facilitating their rapid clonal expansion. Activation of *MET* signaling is a unique resistance mechanism to kinase inhibitors because it can occur through multiple independent mechanisms, amplification or ligand mediated, and when combined can lead to rapid evolution of drug resistance. Our current findings provide insight into future therapeutic strategies for the treatment of *EGFR*-mutant NSCLC. Although *MET* amplification has been detected in up to 20% of NSCLC patients with the *EGFR* mutations who develop acquired resistance to gefitinib or erlotinib, activation of *MET* signaling (by both amplification and mediated by HGF) may in fact account for a larger fraction of gefitinib- or erlotinib-resistant tumors. It is tempting to speculate that HGF production by the stroma may also partially explain why clinical resistance emerges discordantly in some tissues, such as the liver, bone, and brain, whereas pulmonary disease continues to respond to erlotinib treatment (authors' personal observation). Our study further implies that the therapeutic combination of an irreversible EGFR inhibitor (effective against *EGFR* T790M) and a *MET* inhibitor is an attractive treatment combination for a significant portion of patients with gefitinib- and erlotinib-resistant, *EGFR*-mutant NSCLC. In addition, these findings highlight the potential to prospectively identify treatment-naïve patients with *EGFR*-mutant lung cancer who are likely to develop *MET* amplification and may benefit from initial combination therapy with a *MET* inhibitor.

EXPERIMENTAL PROCEDURES

Cell Culture Reagents, Viability Studies, and Western Analyses

Cell lines and growth conditions are described in Supplemental Experimental Procedures. Gefitinib and lapatinib were obtained from commercial sources (American Custom Chemical Corporation and LC Laboratories, Woburn,

MA). PF00299804, PHA-665,752, and PF2341066 were provided by Pfizer (La Jolla, CA). Cell viability was assessed 72 hr following drug exposure by Syto60 staining (Invitrogen) or by MTS assay (Promega). Cells were lysed in an NP-40 containing lysis buffer, separated by SDS-PAGE electrophoresis and transferred to PVDF membranes. Immunoblotting was performed according to the antibody manufacturer's recommendations. Antibody binding was detected using enhanced chemiluminescence (PerkinElmer, Waltham, MA).

Generation of In Vitro Drug-Resistant HCC827 Cells

To generate a resistant cell line, HCC827 cells were exposed to increasing concentrations of PF00299804, similar to our previously described methods (Engelman et al., 2006; Engelman et al., 2007b). PF00299804 concentrations were increased stepwise from 1 nM to 1 μ M when the cells resumed growth kinetics, similar to untreated parental cells. To confirm the emergence of a resistant clone, MTS assays were performed following growth at each concentration.

In Vivo Treatment Studies

All xenograft studies were performed in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee of Massachusetts General Hospital. Generation and treatment of xenograft models were performed as previously described and detailed in Supplemental Experimental Procedures (Engelman et al., 2007a).

SNP Analyses

SNP analyses to evaluate genomewide copy number changes were performed as described elsewhere (Engelman et al., 2007b). Comparison of gene copy number between HCC827 and the PFR clones was performed using dChip software according to previously established methods (Engelman et al., 2007b; Zhao and Vogt, 2008). SNP data are available from the ncbi gene expression omnibus database (accession number, GSE18797).

FISH Probes and Hybridization

Bacterial artificial chromosome (BAC) clones CTD-2257H21 (*EGFR* [7p11.2]) and RP11-95120 (*MET* [7q31.2]) were purchased from Children's Hospital Oakland Research Institute (Oakland, CA). DNA was extracted using a QIAGEN kit (Valencia, CA) and labeled with Spectrum Green- or Spectrum Orange-conjugated dUTP by nick translation (Vysis/Abbott Molecular, Des Plaines, IL). The CEP7 probe (Vysis/Abbott Molecular, Des Plaines, IL) was used according to manufacturer's instructions. Chromosomal mapping and hybridization efficiency for each probe set were verified in normal metaphase spreads (data not shown). Three color FISH assays were performed as described elsewhere (Engelman et al., 2007b).

High Throughput FISH

A Bioview work station with Duet software (Bioview Ltd., Rehovot, Israel) was used to screen for rare *MET*-amplified cells. Automatic scans were performed according to manufacturer's suggested guidelines after setting classification criteria for each FISH probe. Images were captured and classified in an automated fashion and manually reviewed to ensure accuracy. Any unclassified images were manually reviewed and scored. Any cells that could not be scored were excluded from the analysis. Paraffin embedded specimens derived from patients with NSCLC or from xenografts were manually scanned for evidence of *MET* amplification.

Patients with NSCLC

Tumor specimens from gefitinib- or erlotinib-treated patients were obtained from the Dana Farber Cancer Institute/Brigham and Women's Hospital (Boston, MA), Massachusetts General Hospital (Boston, MA), the Chinese University (Hong Kong, China), and Guangdong Provincial People's Hospital (Guangzhou, China) under institutional review board-approved studies. All patients provided written, informed consent. The presence of an *EGFR* mutation in each specimen was confirmed by exon-specific amplification (exons 18–21), followed by direct sequencing, or using the Surveyor endonuclease coupled with denaturing HPLC (DHPLC), fractionation and sequencing (Janne et al., 2006). The *EGFR* T790M mutation was detected using Surveyor endonuclease coupled with DHPLC or an allele-specific PCR (Janne et al., 2006;

Maheswaran et al., 2008). Both methods are capable of detecting the *EGFR* T790M mutation at an allele frequency of 1%–5%. HGF immunohistochemistry was performed as using an anti-HGF 7.2 antibody kindly provided by Dr. George Vander Woude at the Van Andel Institute (see Supplemental Experimental Procedures).

ACCESSION NUMBERS

Coordinates have been deposited in the NCBI gene expression omnibus database with accession code GSE18797.

SUPPLEMENTAL INFORMATION

Supplemental information includes six figures and three tables and may be found with this article online at doi:10.1016/j.ccr.2009.11.022.

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