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Pre-existence 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 lung cancer patients, 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 naïve *EGFR* mutant lung cancer patients who will benefit from initial combination therapy.

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Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are effective clinical therapies for advanced non-small cell lung cancer (NSCLC) patients with *EGFR* activating mutations (Asahina et al., 2006; Inoue et al., 2006; Paz-Ares et al., 2006; Sequist et al., 2008; Tamura et al., 2008). A recent phase III clinical trial demonstrated that patients with *EGFR* mutant NSCLC had superior outcomes with gefitinib treatment compared to standard first line cytotoxic chemotherapy (Mok et al., 2008). However, despite these dramatic benefits from EGFR TKIs in this genetically defined cohort, all of these patients ultimately develop resistance (referred to as acquired resistance herein) 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)).

In addition to these genetic alterations, activation of IGF-1Rβ/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/erlotinib. Further, clinical activity of the irreversible EGFR inhibitor, PF00299804, has been observed in NSCLC patients that have developed acquired resistance to gefitinib/erlotinib (Janne et al., 2008). As an alternative strategy, to delay or avoid the emergence of resistance, there is increased enthusiasm to utilize agents effective against specific resistance mechanisms as initial systemic therapies. For example, PF00299804 is now being assessed in a phase II 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 pre-exists in a subpopulation of cells prior to 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 previously described methods (Engelman et al., 2006; Engelman et al., 2007b). HCC827 cells were exposed to increasing concentrations of PF00299804, starting with 1nM, until they were able to proliferate freely in 1 M PF00299804, which occurred after 6 months of drug selection. This concentration was chosen because it is ~ 1000 fold greater than the IC_{50} for growth inhibition of HCC827 cells and approximately 5 times greater than the serum concentration of PF00299804 observed in NSCLC patients in the phase I 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 as well as downstream PI3K/AKT and ERK signaling is 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 PF2341066 currently undergoing clinical development (Figure 1C, *upper* and data not shown) (Zou et al., 2007). The combination of PF00299804 and a MET inhibitor effectively inhibited the growth of HCC827 PFR cells while neither agent alone led to growth inhibition (Figure 1C, *upper* and data not shown). In addition, the combination of gefitinib and PF2341066 also effectively inhibited the growth of HCC827 PFR cells (Figure 1C, *lower*). 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 due solely to *MET* amplification. We also evaluated the effects of the irreversible EGFR inhibitor PF00299804 and the MET inhibitor PF-2341066 in an HCC827 PFR xenograft model. Treatment with PF00299804 alone was modestly more effective than treatment with PF2341066 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 re-growth has been observed to date in any of the xenografts (after more than 35 weeks off 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 four fold amplification of *MET*, similar to the amplification previously observed in the gefitinib resistant HCC827 (HCC827 GR) cells ((Engelman et al., 2007b) and Figure 2A). All of the PFR clones also had higher levels of MET protein expression (Figure 2B). Genome-

wide 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 (Figure 2C, D). Furthermore, HCC827 PFR and GR cells share single copy losses of 4p, 5q, 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 set 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 hour 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, C).

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 1μM gefitinib or PF00299804 (Figure 3D). HGF also rescued each of these cell lines from TKI-induced cell death after 72 hours (Figure 4A and Figure S1A-E). 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 Figure S1F, G), 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 compared to the other EGFR-driven cell lines that were tested.

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 70ng/mL HGF into the culture medium (data not shown). Further, 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 PF2341066 alone, but only the combination of gefitinib and PF2341066 completely inhibited tumor growth ($p < 0.001$; gefitinib vs. gefitinib/PF2341066; Figure 3E). Indeed, 3 out of 4 mice were cured after 70 days of combined treatment with no evidence of re-growth 70 days after stopping treatment.

Since 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 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). Interestingly, in three of those cell lines (BT-474, HCC827 and H1975), IGF was unable to maintain PI3K/AKT signaling despite potent activation of IGR-1Rβ (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-1Rβ and/or PI3K/AKT activation (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 hours (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, as we observed that IGF maintained PI3K signaling in PC-9 cells for at least 24 hours 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 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 co-precipitating 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 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-phophorylated band at approximately 110kDa.

To more directly assess if 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 pre-exisiting 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 (Figure 6A, B). These stably resistant cells were termed HCC827-50GR (50ng HGF Gefitinib Resistant) cells (Figure 6A). In contrast, HCC827 cells that are not pretreated with HGF, develop gefitinib resistance only after 6 months of gradually increasing 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 (Figure S4D-F 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 to parental cells and maintained MET phosphorylation in the presence of gefitinib (Figure S4B), mimicking *MET* amplified HCC827 GR cells. Therefore, we examined *MET* copy number using fluorescent in situ hybridization (FISH), and found significant *MET* copy number gains in HCC827-50GR cells compared to parental cells (Figure 6C). Quantitative PCR demonstrated a three to four fold amplification of *MET*, similar to the HCC827 GR and PFR cells (data not shown). These results suggest that *MET* 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*). 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*). Further, 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 pre-existing *MET* amplified clone. We analyzed 4237 individual HCC827 cell nuclei using high-throughput fluorescence in situ hybridization (FISH) (Experimental Procedures) and identified 6 cells (0.14%; 6/4237) that harbored significant *MET* copy number gains (Figure 7B, C). 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 out this small population of pre-existing *MET* amplified cells 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 hours, 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 pre-existing *MET* amplified clone in the HCC827 cells (Figure S5B).

Analyses of tumors with acquired resistance to gefitinib/erlotinib reveal evidence of pre-treatment 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 *EGFR* mutant NSCLC patients (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 pre and post geftinib/erlotinib treatment specimens and 11 with drug resistance specimens alone. All specimens, when feasible, were evaluated for *MET* amplification, HGF expression by immunohistochemistry (IHC), and presence of *EGFR* T790M (Figure 8 and Figure S6). We observed *EGFR* T790M in 55 % (15/27) and *MET* amplification in 4/27 (15%) of resistant tumor specimens. In patients with paired tumor specimens, HGF expression was higher in the drug resistant specimens compared to pre-treatment 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 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 pre-treatment specimens for evidence of *MET* amplification. In all 4 patients with *MET* amplification in the drug resistant specimens, we observed rare (< 1%) tumor cells with *MET* amplification from the corresponding pre-treatment specimens (Figure 8A, B). In contrast, of 8 cases that had resistant cancers without *MET* amplification, we observed rare *MET* amplified tumor cells in only 1 of the corresponding pre-treatment tumor specimen. These findings are consistent with cell line data (Figure 7B, C) where we observed evidence of pre-existing *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., 2008; 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 pre-exist 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 *prior* to treatment and undergo clonal selection during imatinib exposure (Hofmann et al., 2003; Roche-Lestienne et al., 2003; Roche-Lestienne et al., 2002; Shah et al., 2002). Similarly, *EGFR* T790M can be detected at low levels in *EGFR* mutant NSCLC patients 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 NSCLC patients that subsequently develop *MET* amplification at the time of clinical gefitinib or erlotinib resistance (Figure 8). The identification of a drug resistance mechanism from a pre-treatment tumor specimen provides the opportunity to specifically target that resistance mechanism prior to its emergence. This approach is clinically appealing as 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., 2008; Paz-Ares et al., 2006; 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/or will provide more clinical benefit than treatment at the time of relapse.

Intriguingly, HCC827 cells appear to be pre-disposed to the development of low level *MET* amplification as 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 pre-determined and occur as a result of drug selection. Understanding why some *EGFR* mutant cancers are pre-disposed 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 *in vitro* and *in vivo*. Unlike in *MET* amplified resistant cancers, HGF mediated resistance occurs through GAB1, not ERBB3, signaling. Higher levels HGF can be detected in tumor specimens from NSCLC patients that are clinically resistant to gefitinib or erlotinib compared to pre-treatment 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 (Figure S6C) and 14) than in the pre-treatment specimen, supporting a role for HGF alone in promoting drug resistance. This is consistent with prior observations (Yano et al., 2008). Ligand mediated drug resistance is unique to HGF as IGF does not rescue TKI-induced cell death in the majority of cell lines tested. Surprisingly, IGF did not restore P13K/AKT signaling in most *EGFR* mutant cancers, despite substantial levels of IGF-1Rβ 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, as 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 as it can occur through multiple independent mechanisms, amplification and/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 *EGFR* mutant patients that 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/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 like the liver, bone and brain, while pulmonary disease continues to respond to erlotinib treatment (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 gefitinib/erlotinib resistant *EGFR* mutant NSCLC patients. In addition, these findings highlight the potential to prospectively identify treatment naïve *EGFR* mutant lung cancer patients 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 hours 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 μM 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 (IACUC) 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 Supplementary Experimental Procedures (Engelman et al., 2007a).

SNP analyses

SNP analyses to evaluate genome wide copy number changes were performed as previously described (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 is 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 RP11-95I20 (*MET* (7q31.2)) were purchased from Children's Hospital Oakland Research Institute (CHORI; 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 previously described (Engelman et al., 2007b).

High throughput fluorescence in situ hybridization

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 NSCLC patients or from xenografts were manually scanned for evidence of *MET* amplification.

NSCLC patients

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 from 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 exonspecific 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).

Highlights

- Rare *MET* amplified cells exist in some *EGFR* mutant lung cancers prior to treatment.
- HGF induces resistance to tyrosine kinase inhibitors in EGFR addicted cancers.

- HGF accelerates *MET* amplification by expanding pre-existing *MET* amplified cells.
- Analysis of pre-treatment cancers identifies those poised to become *MET* amplified.

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 prior to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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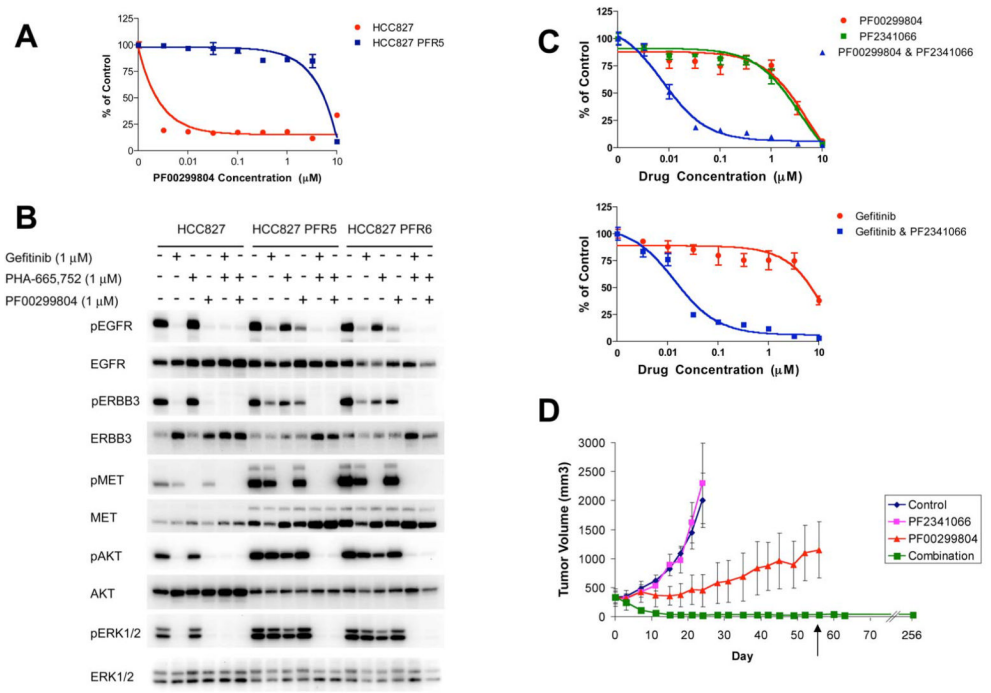


Figure 1. HCC827 PFR cells are resistant to PF00299804, but combined MET and EGFR inhibition blocks PI3K/AKT and ERK signaling and restores sensitivity *in vitro* and *in vivo*

(A) Parental and resistant HCC827 PFR5 cells treated with increasing concentrations of PF00299804. Cell viability relative to untreated controls measured after 72 hours. Each data point represents the mean ±SD of 6 wells. **(B)** HCC827 and HCC827 PFR5 and PFR6 cells were treated for 6 hours with 1 μM PF00299804 or gefitinib, PHA-665,752, or their combination. Cell lysates were immunoblotted to detect indicated proteins. **(C)** *Upper*, HCC827 PFR6 cells treated with increasing concentrations of PF00299804, PF2341066, or their combination. *Lower*, HCC827 PFR6 cells treated with increasing concentrations of gefitinib alone or in combination with PF2341066. Cell viability relative to untreated controls measured after 72 hours. Each data point represents the mean ±SD of 6 wells. **(D)** HCC827 PFR xenogafts 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 re-growth was observed in 35 weeks. Each data point represents the mean ±SD for 5 mice.

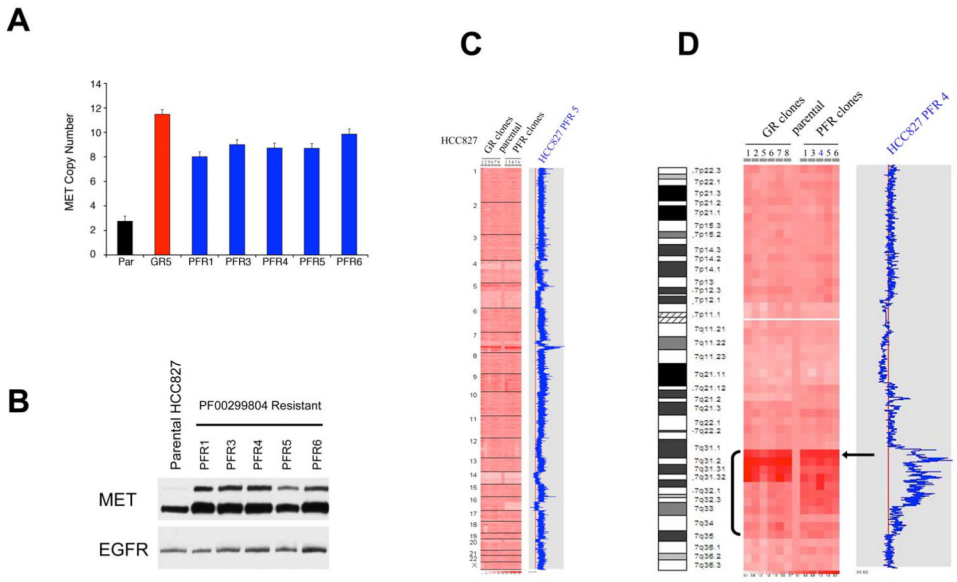


Figure 2. HCC827 PFR cells have a focal amplification in *MET* that is similar to HCC827 GR cells

(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 ±SD for 3 independent experiments. **(B)** Parental HCC827 cells and PFR clones were immunoblotted to detect indicated proteins. **(C)** Genome wide 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.

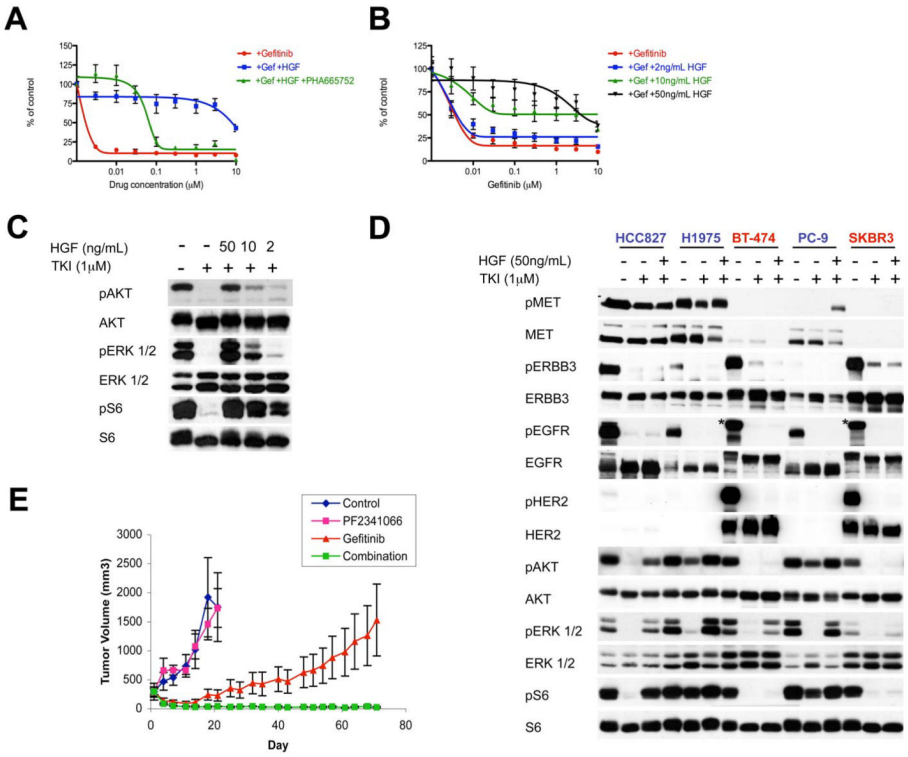


Figure 3. HGF induces MET dependent resistance only in cell lines in which it activates PI3K/AKT, ERK and mTORC1 signaling

(A, B) HCC827 cells treated with (A) increasing concentrations of gefitinib alone or in combination with PHA-665,752, in the absence or presence of HGF (50ng/mL), or (B) increasing concentrations of gefitinib alone or in combination with the indicated concentrations of HGF. Cell viability relative to untreated controls measured after 72 hours. Each data point represents the mean ±SD of 6 wells. **(C)** HCC827 cells were treated for 6 hours with 1μM 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 hours with gefitinib (HCC827, PC-9), PF00299804 (H1975), or lapatinib (BT-474, SKBR3), alone or in combination with HGF (50ng/mL). All drugs were used at 1μM. Cell lysates were immunoblotted to detect indicated proteins. *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 inhibiton was observed with gefitinib alone, however only combination treatment led to complete tumor shrinkage ($p = 0.002$). Each data point represents the mean ±SD for 5 mice. See also Figure S2.

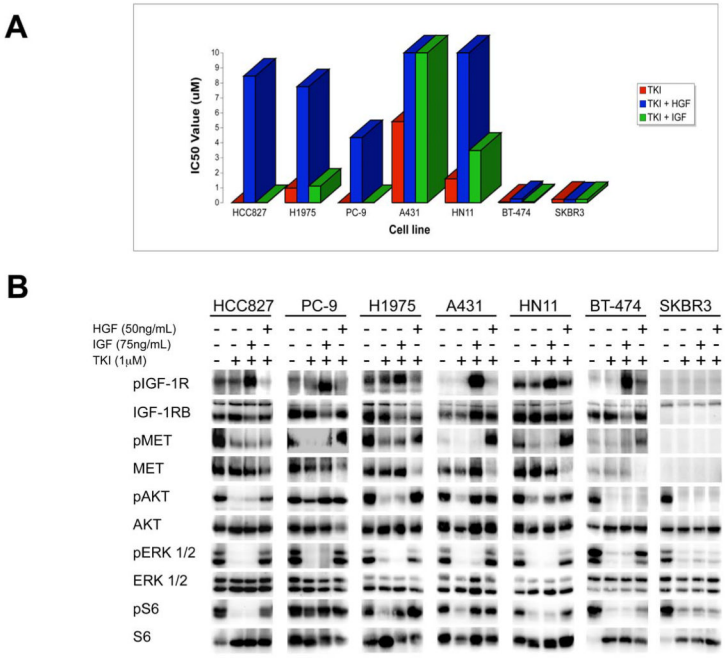


Figure 4. IGF rescues PI3K/AKT and mTORC1 signaling in some cell lines, but fails to activate ERK

(A) IC_{50} 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 50ng/mL HGF (blue) or 75ng/mL IGF (green). **(B)** Cells were treated for 6 hours with gefitinib (HCC827, PC-9, A431, HN11), PF00299804 (H1975) or lapatinib (BT-474, SKBR3) alone or in combination with HGF (50ng/mL) or IGF (75ng/mL). All drugs were used at 1μM. Cell lysates 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.

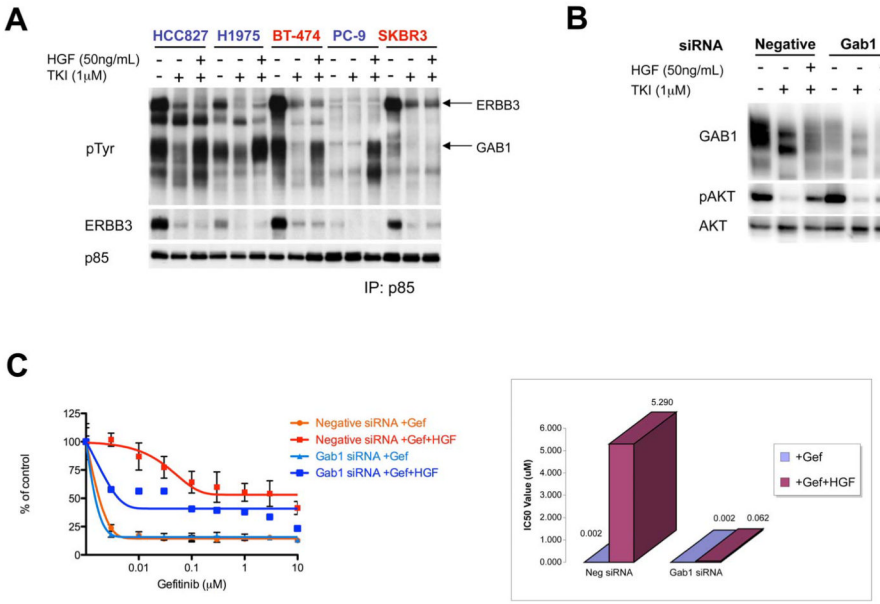


Figure 5. HGF rescue of PI3K/AKT signaling is mediated though GAB1 instead of ERBB3

(A) Cells treated for 6 hours with gefitinib (HCC827, PC-9), PF00299804 (H1975), or lapatinib (BT-474, SKBR3), alone or in combination with HGF (50ng/mL). All drugs were used at 1μM. 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 hours. Transfected cells were treated for 6 hours with gefitinib (1μM) alone or in combination with HGF (50ng/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 hours, then treated with increasing concentrations of gefitinib, alone or in combination with 50ng/mL HGF. *Left*, cell viability relative to untreated controls measured after 72 hours. Each data point represents the mean ±SD of 6 wells. *Right*, plot of IC_{50} values corresponding to cell viability curves.

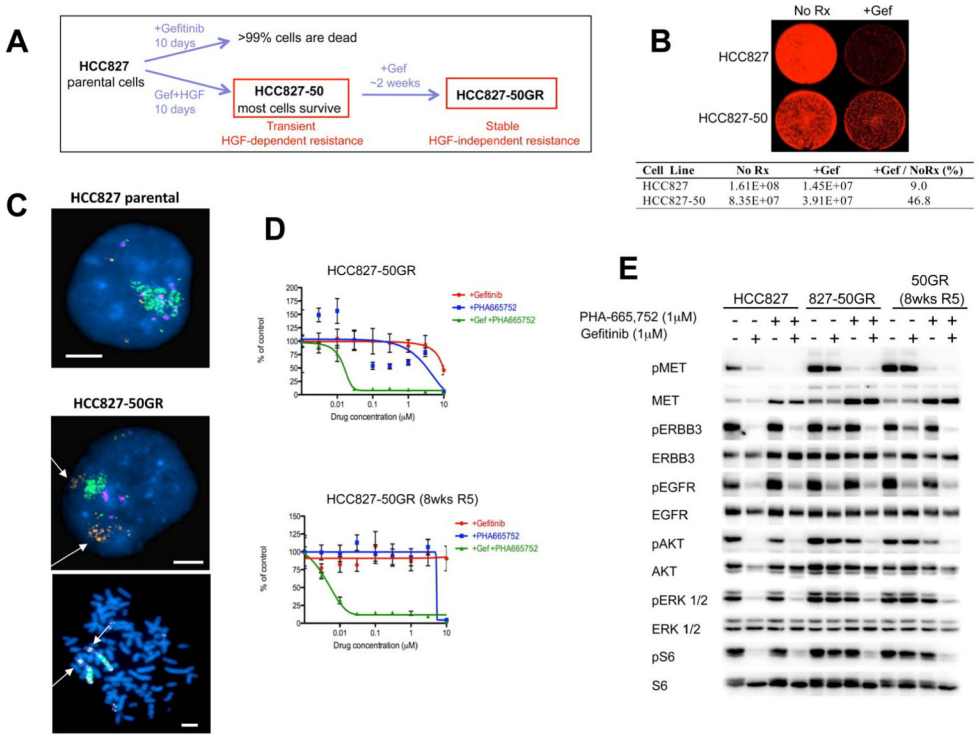
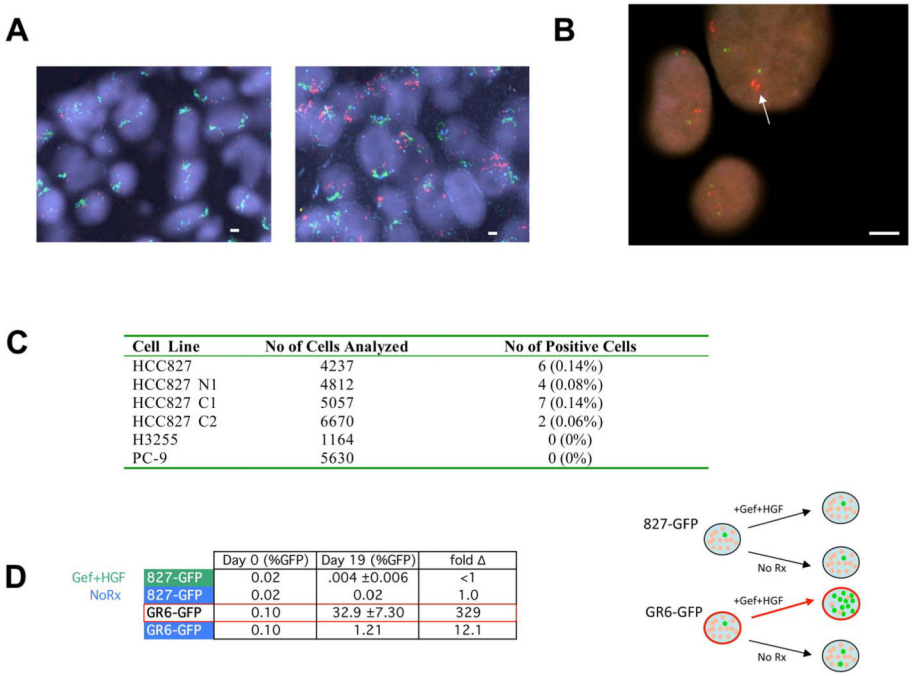


Figure 6. Transient HGF exposure leads to *MET* amplification and stable ligand-independent gefitinib resistance in HCC827 cells

(A) HCC827 cells treated with HGF (50ng/mL) and 1μM gefitinib are resistant to gefitinib (HCC827-50 cells). After the removal of HGF, stably resistant HGF-independent HCC827-50GR cells survive in 1μM gefitinib alone. In contrast, parental HCC827 cells do not survive when treated with 1μM gefitinib. **(B)** Parental HCC827 cells and HCC827-50 cells (pre-treated with gefitinib in combination with HGF (50ng/mL) for 14 days) were grown in media alone (No Rx) or media treated with 1μM 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) *CEP7* (aqua). Metaphase spread (*bottom*) shows multiple copies of *EGFR* and *MET* (arrow) on individual chromosomes. Scale bars represent 10μm. **(D)** HCC827-50GR cells (*upper*) and HCC827-50GR cells grown in media alone (without gefitinib) for 8 weeks, 50GR 8wks R5 (*lower*), were treated with increasing concentrations of gefitinib or PHA-665,752 or their combination for 72 hours. Cell viability was measured relative to untreated controls. Each data point represents the mean ±SD of 6 wells. **(E)** HCC827 cells and stably resistant HCC827-50GR cells were treated for 6 hours with gefitinib, PHA-665,752, or their combination. All drugs were used at 1μM. Cell lysates were immunoblotted to detect indicated proteins. See also Figure S4 and Table S2 and S3.



Cell Line	No of Cells Analyzed	No of Positive Cells
HCC827	4237	6 (0.14%)
HCC827 N1	4812	4 (0.08%)
HCC827 C1	5057	7 (0.14%)
HCC827 C2	6670	2 (0.06%)
H3255	1164	0 (0%)
PC-9	5630	0 (0%)

		Day 0 (%GFP)	Day 19 (%GFP)	fold Δ
Gef+HGF	827-GFP	0.02	.004 ±0.006	<1
NoRx	827-GFP	0.02	0.02	1.0
	GR6-GFP	0.10	32.9 ±7.30	329
	GR6-GFP	0.10	1.21	12.1

Figure 7. HGF treatment selects out a small pre-existing 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) CEP7 (aqua). *Left*, tumor sections from control HCC827 xenograft models that do not express HGF showed normal *MET* copy number. *Right*, tumor sections from one of three HCC827-HGF xenografts treated with gefitinib (Figure 3E) showed significant *MET* amplification *(arrow)*. **(B)** High-throughput FISH analysis of HCC827 cells identifies a subpopulation harboring *MET* amplification *(arrow)*. *MET* (RP-11-95I120; red); 7qter (RP-11-6903; green). All scale bars represent 10μm. **(C)** Parental HCC827 cells and three independent clones harbor a small percentage of *MET* amplified cells. No pre-existing *MET* amplification was detected in H3255 or PC-9 cell populations. **(D)** *Left*, 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 (1μM) with HGF (50ng/mL). Cells were collected after 19 days and GFP levels were quantified using FACS. Each data point for cells treated with gefitinib+HGF represents the mean ±SD for 3 independent wells. Fold change is the ratio of Day 19 to Day 0 (%GFP). *Right*, diagrammatic depiction of results. See also Figure S5.

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
				Exon 19 del	Yes	No	200
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
				Exon 19 del	Yes	No	350
8	L858R	N/A	N/A	L858R	Yes	No	90
9	G719S, S768I	No	95	None*	N/A	No	60
10	L858R	Yes (< 1%)	60	L858R	No	Yes	400
11	Exon 19 deletion	N/A	70	Exon 19 del	Yes	No	300
12	L858R	N/A	100	L858R	No	No	50
13	Exon 19 del	No	300	Exon 19 del	Yes	No	145
14	L858R	No	40	L858R	No	No	180
15	Exon 19 del	Yes (< 1%)	0	Exon 19 del	Yes	Yes	100
16	Exon 19 del	Yes (< 1%)	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

B

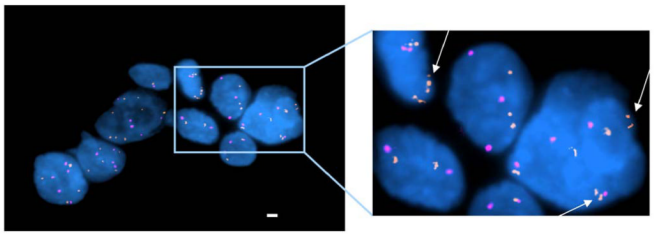


Figure 8. HGF expression and pre-existing *MET* amplification can be detected in tumor specimens from NSCLC patients

(A) Summary of tumors from geftinib/erlotinib treated patients, including 16 paired, and 11 drug resistant samples only. Samples were evaluated for *EGFR* mutational status, *MET* amplification and HGF expression *Specimen contained less < 30% tumor cells. ***MET* amplification defined by qPCR as previously described (Engelman et al., 2007b). Data on *EGFR* T790M and *MET* amplification in resistant specimens only from patients 1-4 and 17-19 has been previously published (Engelman et al., 2007b). N/A; not available. **(B)** FISH analysis of pre-treatment sample from patient 10 shows evidence of a subset of *MET* amplified cells *(arrow)* before exposure to an EGFR TKI. *MET* (RP-11-95I120; orange); *CEP 7* (aqua). Scale bars represent 10μm. See also Figure S6.