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Acquired Resistance of Non–Small Cell Lung Cancer Cells to MET Kinase Inhibition Is Mediated by a Switch to Epidermal Growth Factor Receptor Dependency

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Abstract

Cancer cells harboring *MET* amplification display striking sensitivity to selective small molecule inhibitors of MET kinase, prompting their clinical evaluation. Similar to the experience with traditional therapeutics, most patients responding to treatment with such molecular targeted therapeutics ultimately relapse with drug-resistant disease. In this study we modeled acquired resistance to experimental MET kinase inhibitor PF2341066 in *MET*-amplified non–small cell lung carcinoma (NSCLC) cell lines to identify drug resistance mechanisms that may arise in clinic. We found that activation of the epidermal growth factor receptor (EGFR) pathway emerges as a resistance mechanism in *MET*-amplified cells after prolonged exposure to PF2341066. Whereas combined inhibition of MET and EGFR kinases in MET-dependent NSCLC cells did not enhance their initial sensitivity to PF2341066, this combination dramatically suppressed the eventual emergence of drug-resistant clones after prolonged drug exposure. Conversely, activation of the EGFR pathway increased the yield of PF2341066-resistant clones, confirming the significance of this pathway in conferring resistance. Our findings support an intimate relationship between the EGFR and MET signaling pathways in NSCLC, and they suggest that combination treatment with MET and EGFR kinase inhibitors may be beneficial in *MET*-amplified NSCLC by reducing selection for drug resistant clones. *Cancer Res; 70(4); 1625–34. ©2010 AACR.*

Introduction

Many human receptor tyrosine kinases (RTK) mediate signals that promote proliferation, migration, and survival of cancer cells. Consequently, RTKs have been long recognized as potentially important targets for the development of cancer therapeutics, and ~30 distinct kinase inhibitors have been developed thus far to the level of Phase I clinical evaluation (1). The clinical success of tyrosine kinase inhibitors (TKI), such as erlotinib and imatinib, has prompted intensive efforts to identify and target additional oncogenic kinases as a broad therapeutic strategy for selected patient populations (2–5).

Focal amplification of the *MET* gene, which encodes the MET RTK, is detected in a subset of solid tumors—most notably, gastric and non–small cell lung carcinoma (NSCLC) cancers (6, 7). In NSCLC cell lines, amplified *MET* is associated with active MET kinase and consequent engagement of established MET effectors, such as AKT and extracellular signal-regulated kinases 1/2 (ERK1/2; ref. 8). Furthermore, MET kinase inhibition in *MET*-amplified cancer cells results in decreased cancer cell viability in cell culture and xenografts models, suggesting that MET TKIs may be clinically effective in patients whose tumors harbor *MET* amplification (8–11). Consequently, MET TKIs are currently undergoing early-phase clinical testing as anticancer agents.[4]

Although the clinically approved TKIs can yield impressive responses in a subset of treated cancer patients, rapidly acquired drug resistance remains an important limitation to the long-term efficacy of such treatments (12–15). Therefore, it is critical to establish mechanisms of drug resistance and to apply that knowledge to the development of strategies to combat resistance (13, 16–18). One such strategy is to treat tumors with a combination of agents that might prevent the emergence of drug-resistant cells by anticipating specific mechanisms of resistance that might otherwise arise in the context of single agent–based therapies.

Here, we established preclinical findings suggesting that acquired resistance to MET TKIs in MET-dependent NSCLC

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cells is associated with either a partial or complete switch to epidermal growth factor (EGF) receptor (EGFR)–dependent signaling to maintain tumor cell survival. Despite the absence of any detectable sensitivity to EGFR TKIs in these NSCLC cell lines, combined MET/EGFR kinase blockade dramatically suppresses the emergence of drug-resistant clones, pointing to a potential therapeutic strategy to reduce the likelihood of relapse in the treatment of NSCLC patients with *MET*-amplified tumors and highlighting an intimate relationship between MET and EGFR signaling in NSCLC.

Materials and Methods

Human cancer cell lines and cell viability assays. The EBC-1 and NCI-H1993 cell lines were from the Japanese Health Sciences Foundation (JHSF) and the American Type Culture Collection (ATCC), respectively, and cultured according to their recommendations. COR-L 105 cells were from the European Collection of Animal Cell Cultures. EBC-1 cells were authenticated by JHSF using short tandem repeat DNA profiling. ATCC has not published a DNA STR profile for NCI-H1993, so a sample was sent to the Wellcome Trust Sanger Institute for single-nucleotide polymorphism analysis and comparison with their cancer cell line collection. The only match was with NCI-H1993 in that collection. COR-L 105 cells were similarly genotyped by the Wellcome Trust Sanger Institute and found to match their COR-L 105 stock and no other lines. All cell lines were obtained directly from each repository and passaged for fewer than 6 mo after receipt. All drug-resistant clones were removed from drug for a minimum of 10 d before undergoing cell viability assays. Cell viability was measured as previously described (10). In brief, cells were fixed in 4% formaldehyde and incubated in the fluorescent DNA-binding dye Syto-60 (Invitrogen) before analysis on a fluorescent plate reader. Sensitivity to drug treatment was calculated as the fraction of viable cells relative to untreated cells after a 72-h exposure. Data were subjected to nonlinear regression analysis using GraphPad Prism Software version 5.2 (GraphPad Software, Inc.) to obtain IC_{50} values.

Generation of PF2341066-resistant clones. Drug-resistant clones of the PF2341066-sensitive *MET* amplified NSCLC cell lines EBC-1 and NCI-H1993 were established by exposing these cells to increasing concentrations of PF2341066 for 3 mo. Clones capable of proliferation in a final concentration of 1 μmol/L were selected, for subsequent experiments were transferred to separate plates and treated with 1 μmol/L of PF2341066 every 2 wk thereafter. The drug-resistant cells were designated PR (PF2341066 resistant).

Protein detection. Immunodetection of proteins after SDS-PAGE was performed using standard protocols. Equal lane loading was assessed using β-tubulin (Sigma) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon) antibodies. The AKT, ERK1/2, phospho-ERK1/2 (T^{202}/Y^{204}), MET, phospho-MET ($Y^{1234/35}$), signal transducers and activators of transcription 3 (STAT3), and phospho-STAT3 (S^{727}) antibodies were from Cell Signaling Technology. The phospho-AKT (S^{473}) antibody was from BioSource International. The phospho-EGFR antibody was from Abcam. The poly(ADP-ribose) polymerase and total EGFR antibodies were from BD Biosciences. All antibodies were used at a 1:1,000 dilution, except for the β-tubulin and GAPDH antibodies, which were used at 1:10,000 dilution.

Kinase inhibitors. PHA665752 was synthesized at the Dana-Farber Cancer Institute (DFCI), and PF2341066 was synthesized by Pfizer Pharmaceuticals. All compounds were reconstituted in DMSO to a 10 mmol/L concentration and stored at −80°C. Additional compounds detailed in the supplementary tables were synthesized at DFCI or obtained through commercial suppliers.

Giemsa staining of drug-resistant colonies. Plates of cultured cells were washed in PBS before adding ice-cold methanol as a fixative. After fixation cells were incubated in Giemsa stain (Sigma-Aldrich) for 1 h before washing in distilled water and air drying. Colonies containing >50 cells were counted under microscopy.

Human growth factor antibody array. Cells were seeded at equivalent density in six-well plates, and the following day the medium was replaced with 1 mL serum-free medium. Twenty-four hours later conditioned medium was incubated with the antibody array as per the manufacturer's instructions (RayBiotech, Inc.). Detection of signal was achieved using horseradish peroxidase–conjugated streptavidin and exposure of the array membrane to X-ray film.

Results

Establishment of* MET*-amplified NSCLC cells with acquired resistance to a selective MET kinase inhibitor. The NSCLC cell line EBC-1 was previously shown to harbor focal *MET* gene amplification and is exquisitely sensitive to inhibition of MET kinase activity (Fig. 1A; ref. 10). To explore potential mechanisms of acquired resistance to MET-targeted therapeutics, we used the selective small molecule MET TKI PF2341066, which is currently being evaluated in clinical trials.[4] PF2341066-resistant clones were generated from EBC-1 cells by exposing cells to increasing concentrations of PF2341066 for 3 months. The resultant clones (designated EBC-1 PR1 to EBC-1 PR22), following expansion from single cells, showed resistance to PF2341066 treatment, with the majority of clones exhibiting at least 50-fold reduced drug sensitivity and several clones showing >250-fold reduced drug sensitivity (Fig. 1A; Supplementary Table S1). The drug-resistant clones also exhibited cross-resistance to an additional selective MET TKI, PHA665752 (Fig. 1A; Supplementary Table S1).

To explore the biochemical basis for acquired MET TKI resistance in the EBC-1–derived clones, the phosphorylation state of MET and the downstream effectors AKT and ERK1/2 was examined. As expected, in the parental EBC-1 cells, acute treatment with PF2341066 causes virtually complete suppression of MET autophosphorylation, as well as phosphorylation of AKT and ERK1/2 (Fig. 1B). Similarly, in each of nine independent EBC-1–derived drug-resistant clones, PF2341066 treatment caused suppression

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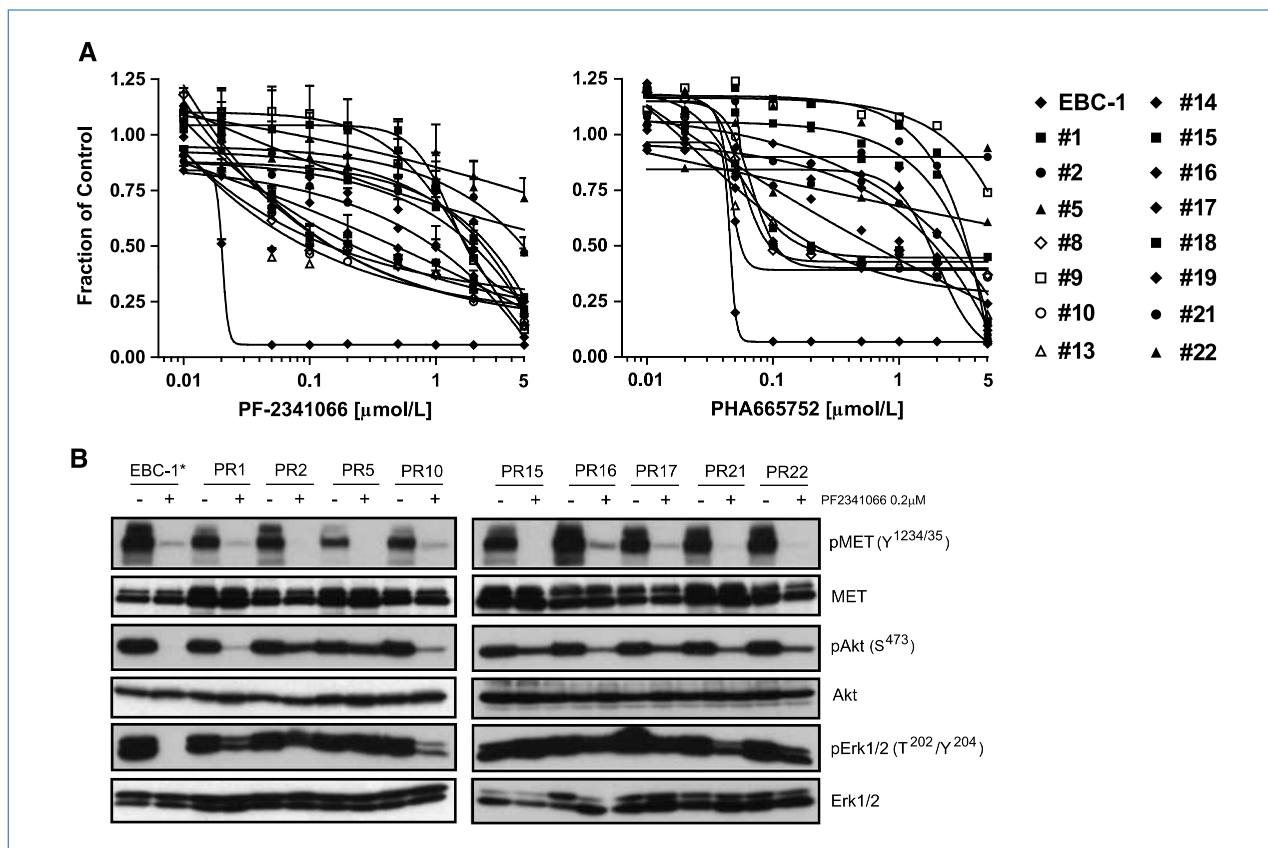

Figure 1. Generation of PF2341066-resistant NSCLC cells. A, PF2341066-resistant clones derived by exposing the EBC-1 NSCLC cell line to increasing concentrations of the MET TKI PF2341066 weekly for 3 mo exhibit increased resistance to PF2341066 as well as an additional MET TKI, PHA665752. The 15 drug-resistant clones are designated as "PR" (PF2341066 resistant). Cell viability was assayed 72 h after treatment with the indicated drug concentrations. Independent triplicate tests were performed, and the results reflect the mean and SD. B, PF2341066-resistant cells maintain AKT and ERK1/2 phosphorylation in the presence of PF2341066. EBC-1 and EBC-1 PR cells were treated for 6 h with 200 nmol/L PF2341066. Cell extracts were immunoblotted to detect the indicated proteins. Total proteins are shown to serve as loading controls. The parental cell line EBC-1 is indicated by an asterisk (*).

of MET phosphorylation, indicating that the observed drug resistance does not reflect drug efflux or a mutation that prevents drug binding to MET. However, in contrast to the findings with parental EBC-1 cells, complete inhibition of phosphorylation of AKT and ERK1/2 in the drug-resistant clones was not observed after PF2341066 treatment (Fig. 1B), suggesting an uncoupling of these critical cell survival effectors from MET kinase function in the drug-resistant clones.

Acquired resistance to MET kinase inhibition is associated with a switch to EGFR dependency. To identify cell survival pathways specifically engaged in the EBC-1–derived PF2341066-resistant cells, clones PR5 and PR22 (>250-fold increased resistance to PF2341066) were tested for sensitivity to 35 established and investigational anticancer agents (Supplementary Table S2) in a 72-hour cell viability assay (Fig. 2A, B). Among the tested agents, EBC-1 PR5 cells displayed substantial sensitivity to two different EGFR TKIs, erlotinib and HKI-272 (Fig. 2A), as single agents, whereas EBC-1 PR22 cells displayed sensitivity to both of these compounds only when applied in combination with PF2341066 (Fig. 2B). These results suggest that EBC-1 PR5 cells have completely switched their survival dependency from MET to EGFR kinase-mediated signaling, whereas the EBC-1 PR22 cells seem to have become codependent on both MET and EGFR signaling to maintain cell survival. Significantly, the erlotinib had no effect on ERK1/2 or AKT signaling pathways in the EBC-1 parental cell line (Fig. 2C). In contrast, erlotinib treatment of the EBC-1 PR5 clone completely abolished ERK1/2 signaling (Fig. 2D, lanes 3–6). Suppression of downstream signaling was similarly seen in EBC-1 PR22, but only when erlotinib and PF2341066 were tested in combination (Fig. 2D, lanes 10 and 12).

To determine whether a switch to either partial or complete EGFR dependency occurs in the context of acquired resistance to PF2341066 in the other drug-resistant clones, each clone was treated with erlotinib alone or in combination with PF2341066 and cell viability was measured. Thirteen (87%) of the 15 tested EBC-1 PR clones showed acquisition of sensitivity either to single-agent erlotinib or to the combination of erlotinib and PF2341066 (Supplementary Fig. S1A, B), suggesting that in these *MET*-amplified cells EGFR-mediated

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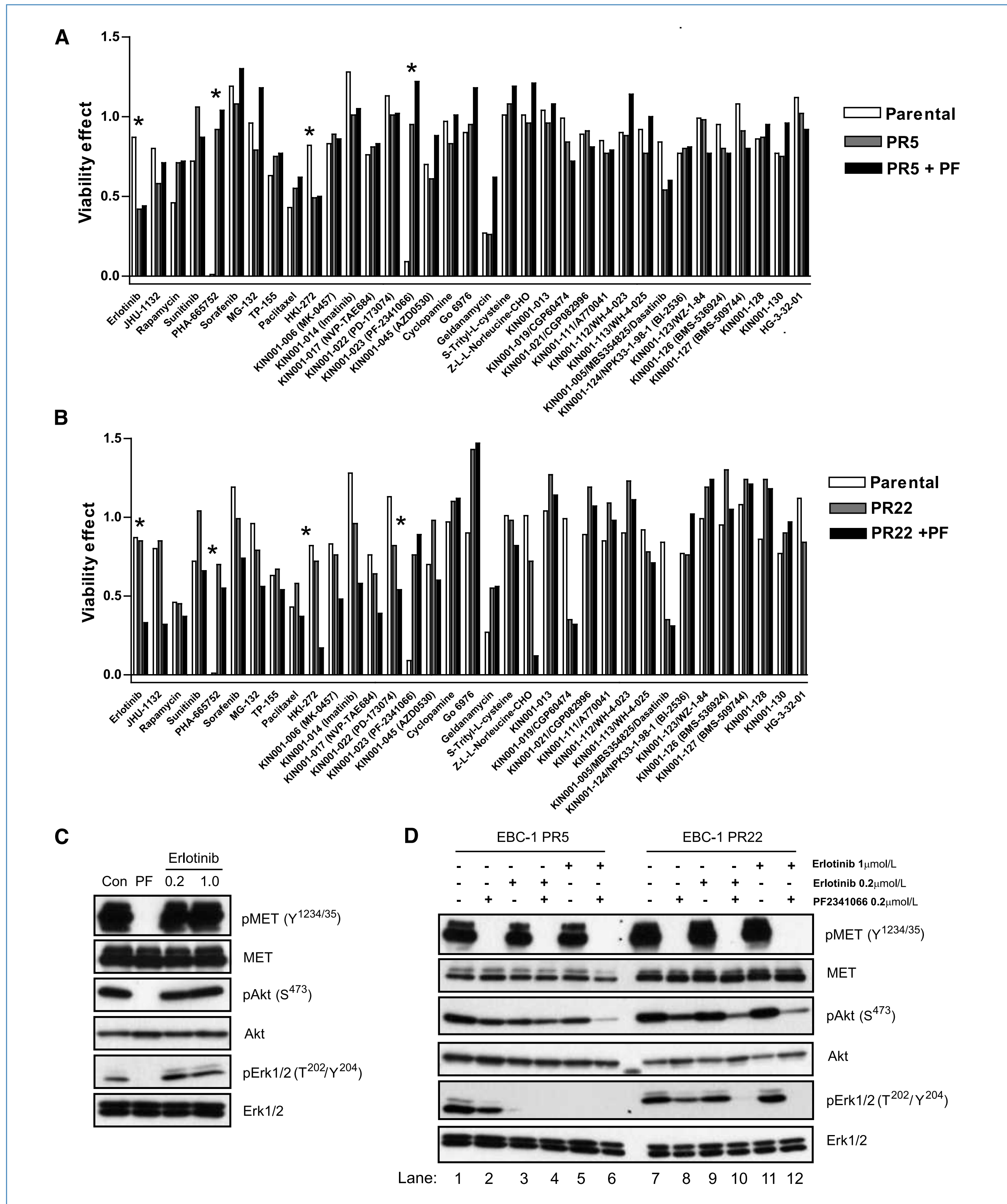

Figure 2. Engagement of EGFR-dependent survival signaling in PF2341066-resistant NSCLC cells. The parental EBC-1 cell line and MET TKI-resistant clones PR5 (A) and PR22 (B) were treated with the 35 indicated established or investigational anticancer compounds in the absence and presence of PF2341066 (0.2 μmol/L; PF), and cell viability was measured after 72 h. Asterisks indicate results of particular interest. C, the parental EBC-1 cell line was treated with either PF2341066 (0.2 μmol/L) or erlotinib (0.2 or 1 μmol/L) for 6 h. Cell extracts were immunoblotted to detect the indicated proteins. D, MET TKI-resistant EBC-1 clones PR5 and PR22 were treated for 6 h with either PF2341066 (0.2 μmol/L), erlotinib (0.2 or 1 μmol/L), or both in combination. Cell extracts were immunoblotted to detect the indicated proteins. Total proteins serve as normalization controls.

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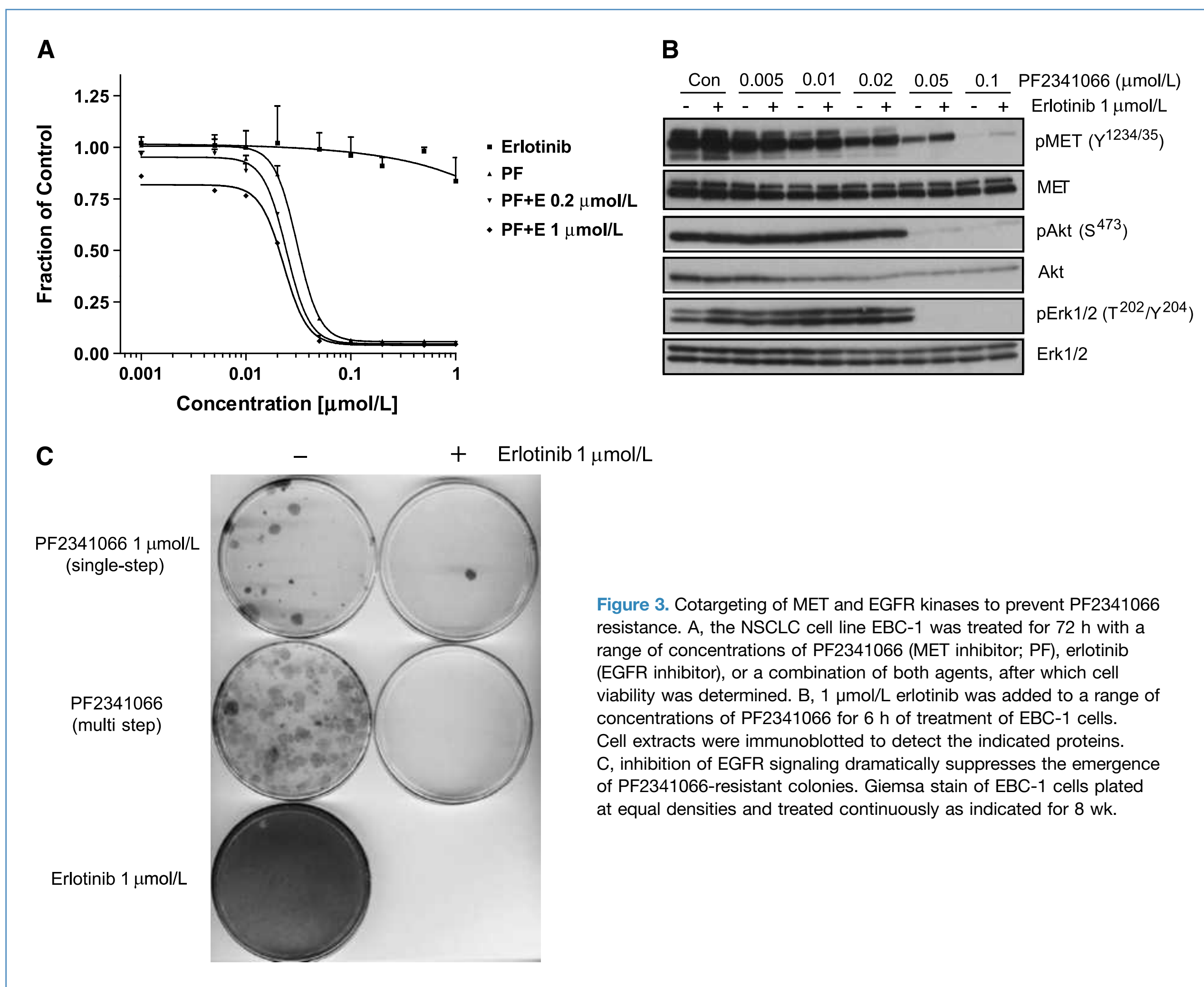

Figure 3. Cotargeting of MET and EGFR kinases to prevent PF2341066 resistance. A, the NSCLC cell line EBC-1 was treated for 72 h with a range of concentrations of PF2341066 (MET inhibitor; PF), erlotinib (EGFR inhibitor), or a combination of both agents, after which cell viability was determined. B, 1 µmol/L erlotinib was added to a range of concentrations of PF2341066 for 6 h of treatment of EBC-1 cells. Cell extracts were immunoblotted to detect the indicated proteins. C, inhibition of EGFR signaling dramatically suppresses the emergence of PF2341066-resistant colonies. Giemsa stain of EBC-1 cells plated at equal densities and treated continuously as indicated for 8 wk.

signaling is a potent and "preferred" mechanism for maintaining cell survival when the population is chronically exposed to a MET TKI. Notably, there was no evidence of increased EGFR expression in any of the drug-resistant clones compared with parental EBC-1 cells (Supplementary Fig. S1C).

Combined MET and EGFR blockade prevents the emergence of drug resistant clones. The observed switch to EGFR dependency or the acquisition of codependency on MET and EGFR signaling in the context of MET TKI resistance prompted us to examine the potential benefit of combining these inhibitors to prevent the emergence of drug-resistant NSCLC cells. First, we established that the combination of erlotinib and PF2341066 does not detectably decrease the IC_{50} value relative to PF2341066 treatment alone in the setting of EBC-1 cells, as measured by growth inhibition (Fig. 3A). Moreover, combined MET and EGFR kinase blockade did not yield any enhanced effect on suppression of AKT and ERK1/2 activation in these cells (Fig. 3B). We then generated PF2341066-resistant clones as described above by exposing the cells to increasing PF2341066 concentrations ("multistep") or by exposing cells weekly to a 1 µmol/L concentration ("single-step"), either in the presence or absence of 1 µmol/L erlotinib. Erlotinib alone had no effect on cell viability, but the addition of erlotinib to PF2341066 almost completely inhibited the development of PF2341066-resistant clones (Fig. 3C).

To further explore the role of EGFR activation in the development of resistance to a MET kinase inhibitor, EBC-1 cells were exposed over 4 weeks to PF2341066 in the presence of the EGFR ligand, EGF. In the acute treatment setting, combined exposure to supplementary EGF and PF2341066 had almost no effect on short-term viability of EBC-1 cells (Supplementary Fig. S2A); however, addition of EGF to PF2341066-treated cells led to a persistent low level of ERK1/2 phosphorylation despite the complete suppression of MET autophosphorylation (Supplementary Fig. S2B). Furthermore, the combination of supplementary EGF and PF2341066 yielded a significant increase in the number of PF2341066-resistant clones generated after chronic drug exposure (Supplementary Fig. S2C). These findings

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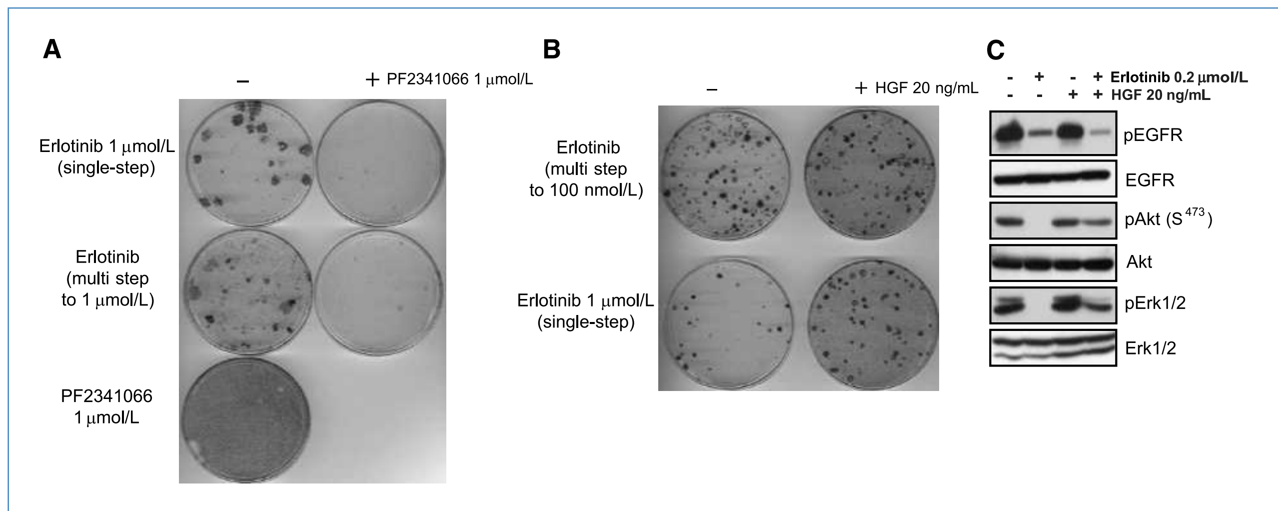

Figure 4. Combined EGFR and MET blockade inhibits the development of drug-resistant clones in *EGFR* mutant NSCLC cells (HCC827). A, Giemsa stain of HCC827 cells plated at equal densities and treated continuously as indicated for 8 wk. B, Giemsa stain of HCC827 cells plated at equal densities and treated as indicated for 8 wk with erlotinib and HGF weekly. C, immunoblot analysis of extracts of HCC827 cells 6 h after the indicated treatments to detect the phosphorylation status of EGFR, AKT, and ERK1/2. Total proteins serve as normalization controls.

further support a role for EGFR signaling in the emergence of MET TKI resistance in MET-dependent NSCLC cells and suggest that ligand-mediated EGFR activation could constitute a potential mechanism.

To further explore a potential role for ligand-dependent activation of EGFR in this setting, a membrane-based antibody array was used to compare the expression of 41 growth factors in conditioned medium prepared from the parental EBC-1 cell line and the PF2341066-resistant clone PR22 (Supplementary Fig. S3). This array included three well-established EGFR ligands—EGF, amphiregulin, and transforming growth factor α. This analysis revealed significantly increased expression of amphiregulin in the PR22 cell line compared with EBC-1, suggesting that dysregulated amphiregulin expression may contribute to the acquisition of EGFR dependency in the context of resistance to MET TKI treatment.

Cooperation between the EGFR and MET signaling pathways has also been shown to underlie the acquisition of resistance to EGFR TKI treatment in the setting of the EGFR TKI-sensitive *EGFR* mutant NSCLC cell line HCC827, as well as in *EGFR* mutant NSCLC patients (16). Increased MET signaling, associated with *MET* gene amplification, was found to yield acquired resistance to an EGFR TKI by driving ERBB3-dependent activation of phosphoinositide 3-kinases (PI3K) signaling. Therefore, to determine whether we could prevent the emergence of resistance to EGFR TKI treatment in the setting of MET-amplified NSCLC, using a similar combination strategy to that used with the EBC-1 cell line, we exposed HCC827 cells to either single-step (1 μmol/L) or multistep (5 nmol/L to 1 μmol/L) erlotinib treatment over 8 weeks and in the absence and presence of PF2341066 (1 μmol/L). HCC827 cells are resistant to PF2341066 treatment, and no additive effect was observed when it was included in combination with erlotinib (Supplementary Fig. S4A). Furthermore, there was no detectable effect on prosurvival signaling in HCC827 cells with this combination (Supplementary Fig. S4B). However, the addition of PF2341066 to erlotinib almost completely inhibited the establishment of erlotinib-resistant HCC827 clones (Fig. 4A). Conversely, MET activation with its ligand hepatocyte growth factor (HGF) in erlotinib-treated HCC827 cells (either single-step or multistep) led to an increased number of EGFR TKI-resistant clones after 8 weeks of treatment (Fig. 4B). Furthermore, addition of HGF to HCC827 cells previously treated with erlotinib dramatically reversed the inhibition of AKT and ERK1/2 phosphorylation seen after treatment with erlotinib alone (Fig. 4C). It was recently reported that HGF induces resistance in HCC827 cells to the EGFR TKI gefitinib, and elevated HGF levels were detected in clinical lung adenocarcinoma cases showing intrinsic or acquired resistance to that agent (19). Together, these findings point to an intimate relationship between EGFR and MET signaling in RTK-addicted NSCLC cells and suggest that maintaining signaling through AKT or ERK1/2 via cross-talk between these receptors may be a common mechanism by which RTK-addicted cells are able to survive treatment with small molecule inhibitors of these oncogenic kinases.

To determine whether the EGFR-mediated resistance mechanisms we observed in the *MET*-amplified EBC-1 cell line are more broadly relevant in NSCLC cells, PF2341066-resistant clones were generated using another NSCLC cell line harboring *MET* amplification, NCI-H1993, which is similarly dependent on MET signaling for survival (Fig. 5A; ref. 8). As with EBC-1 cells, treatment of the TKI-resistant clones with PF2341066 for 6 hours completely suppressed MET autophosphorylation in NCI-H1993 PR cells; however, relative to effects observed in the parental NCI-H1993 cell line, there was either partial or complete persistence of ERK1/2 phosphorylation in

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the TKI-resistant cells (Fig. 5B). Furthermore, as in the EBC-1 model, whereas the addition of the EGFR TKI erlotinib to PF2341066 did not significantly affect cell viability in the parental cell line, the combination treatment dramatically reduced the number of PF2341066-resistant clones detected after 8 weeks (Fig. 5C, D). This finding suggests that EGFR-mediated signaling may be a broadly relevant mechanism of acquired MET TKI resistance in the setting of *MET*-amplified NSCLC.

De novo ***resistance to MET TKI treatment in a NSCLC cell line harboring*** **MET** ***gene amplification.*** Acquired resistance to EGFR TKI treatment in the setting of *EGFR* mutant NSCLC has been associated with two distinct genetic mechanisms-acquisition of a secondary T790M mutation within the EGFR kinase domain and focal amplification of the *MET* gene (16, 20). Significantly, both of these genomic alterations have also been observed in some cases of NSCLC showing *de novo* resistance to treatment, suggesting that they may contribute to oncogenicity as well as drug resistance (2, 21). *De novo* drug resistance mechanisms in cancer cell line models have been shown in some cases to be predictive of acquired drug resistance in clinical populations (20).

We previously analyzed genomic single-nucleotide polymorphism array data for a large panel of cancer cell lines, many of which had been tested for PF2341066 sensitivity, and this data set revealed a NSCLC cell line with elevated *MET* copy number but which was resistant to MET TKI treatment (10). This cell line (NCI-H1573) shows *MET* gene amplification and substantial MET phosphorylation. However, unlike with the EBC-1 cell line, PF2341066 treatment had no significant effect on cell viability and did not result in complete inhibition of AKT and ERK1/2 signaling, despite completely suppressing MET autophosphorylation (Supplementary Fig. S5A, B). These findings resemble those seen with the PF2341066-resistant EBC-1 clones, and intriguingly, a comparison of gene copy number changes across 360 "cancer genes" revealed that NCI-H1573 cells harbor both *MET* and *EGFR* amplification (Supplementary Table S3). However, unlike what was observed in a subset of the MET TKI-resistant EBC-1 clones, there was no detectable effect on cell viability when NCI-H1573 cells were treated with erlotinib, either alone or in combination with PF2341066 (Supplementary Fig. S5A). In addition, neither erlotinib alone nor in combination with PF2341066 yielded

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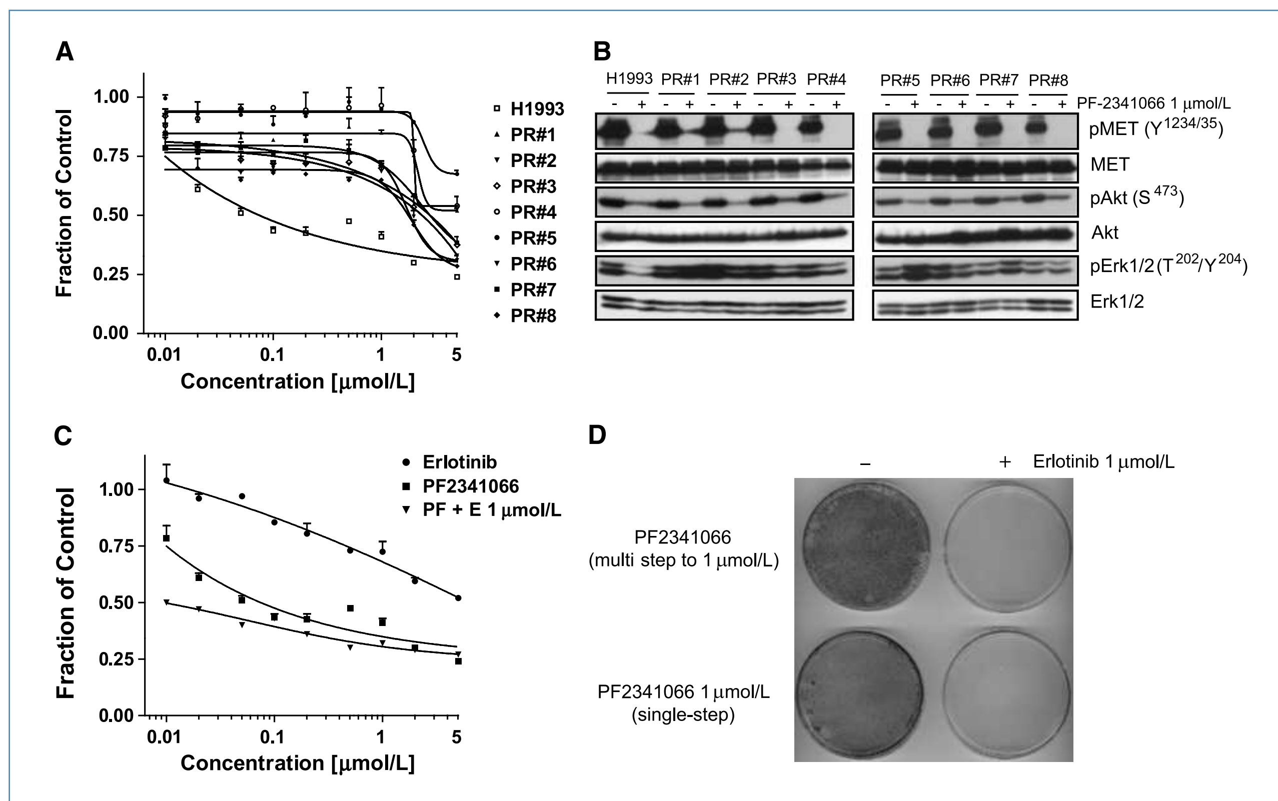

Figure 5. Combined MET and EGFR blockade suppresses the emergence of drug-resistant clones in the *MET*-amplified NSCLC cell line NCI-H1993. A, PF2341066-resistant clones of NCI-H1993 were derived by treating cells with increasing concentrations of PF2341066 weekly for 3 mo (to a final concentration of 1 µmol/L). Cell viability assays reflect 72 h treatments with the indicated concentrations of PF2341066. B, immunoblots showing the effect of PF2341066 treatment on signaling pathways in the parental NCI-H1993 cell line versus PF2341066-resistant clones at a 6-h time point. C, NCI-H1993 cells were treated for 72 h with a range of concentrations of PF2341066, erlotinib (EGFR inhibitor), or a combination of both agents, and the effect on cell viability was measured. D, NCI-H1993 cells were treated weekly (for 8 wk) with either a steadily increasing concentration (starting at 10 nmol/L, ending at 1 µmol/L) or a fixed concentration (1 µmol/L) of PF2341066. In addition, erlotinib (1 µmol/L) was added weekly to similarly treated plates. All plates were fixed and Giemsa-stained at the end of the experiment, and representative plates are shown.

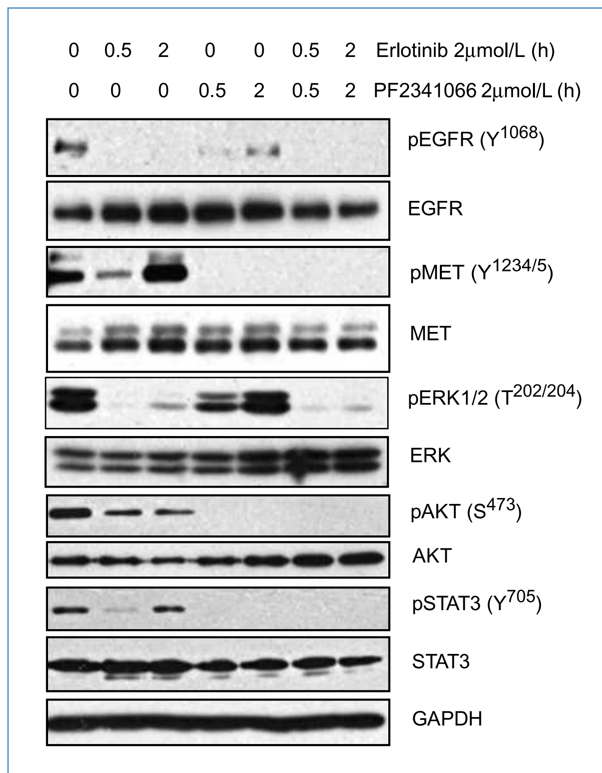

Figure 6. Combined inhibition of EGFR and MET activation disrupts survival signaling in the NSCLC cell line COR-L 105. Immunoblot analysis of COR-L 105 cell lysates at 0.5 and 2 h posttreatment with the indicated TKIs to detect phosphorylated forms of EGFR, MET, AKT, ERK1/2, and STAT3. Total proteins served as normalization controls with GAPDH providing an additional loading control.

complete suppression of AKT and ERK1/2 signaling (Supplementary Fig. S5B).

NCI-H1573 cells were then exposed to an additional 30 compounds known to target critical oncogenic signaling pathways (Supplementary Table S4), either as single agents or in combination with either PF2341066 or PF2341066/erlotinib, to determine whether inhibition of MET and EGFR signaling combined with disruption of a third pathway could inhibit the growth of these cells. However, there was no additional inhibitory effect seen with any of these 30 compounds with the addition of either PF2341066 or the PF2341066/erlotinib combination (Supplementary Fig. S5C). These findings implicate additional as yet unidentified mechanisms of resistance to MET TKIs in some NSCLC cells treated with these targeted therapeutics.

Potential benefit of combining EGFR and MET TKIs in NSCLCs with resistance to single agents. To further explore the potential utility of combining MET and EGFR TKIs in the treatment of NSCLC, we tested a panel of 100 NSCLC-derived cell lines for sensitivity to erlotinib, PF2341066, or a combination of the two inhibitors. Whereas the observed sensitivity to single-agent treatments in the various cell lines tested was well correlated with genomic activation of the target kinase, as expected, a few cell lines lacking *EGFR* or *MET* genomic activation were largely refractory to single-agent treatment but showed notable sensitivity to the combination treatment (Supplementary Table S5). To explore mechanisms underlying sensitivity to the combination treatment, we focused on one of these lines, COR-L 105. By examining the signaling consequence of single or combination TKI treatment of these cells, we determined that inhibition of either EGFR or MET kinase activity results in only partial inhibition of prosurvival signaling pathways (Fig. 6). However the combined inhibition of EGFR and MET signaling led to virtually complete suppression of these pathways. Thus, phosphorylation of ERK1/2 is clearly coupled to EGFR signaling, whereas the phosphorylation of AKT and STAT3 is coupled to MET signaling (Fig. 6). However, unlike in the HCC827 line, resistance to MET TKI is not coupled to ERBB3-dependent activation of PI3K signaling (data not shown). Interestingly, erlotinib-induced inhibition of EGFR phosphorylation leads to an acute decrease in phospho-MET levels (at 0.5 hour posttreatment) followed by an increase in activated MET at 2 hours of treatment (Fig. 6). Similarly, PF2341066-mediated inhibition of MET phosphorylation initially caused a decrease in phospho-EGFR levels at 0.5 hours followed by an increase at 2 hours posttreatment. These findings highlight the complexity of cross-talk between MET and EGFR RTKs in NSCLC, potentially involving feedback regulation after TKI treatment.

Discussion

Acquired resistance to TKIs in cancer therapy remains a critical limitation to the efficacy of these agents. Despite the clinical success of several of these drugs, the eventual and often rapid development of drug resistance demands a more thorough understanding of the underlying mechanisms to develop effective strategies to manage or prevent drug resistance. Cancer cell lines can provide a powerful model system to identify clinically relevant mechanisms underlying acquired drug resistance (13). Here, we have undertaken this strategy to anticipate potential mechanisms of acquired resistance to MET TKIs, which are currently undergoing clinical testing. Amplified *MET* is observed in ~4% of NSCLCs and 15% of gastric cancers (6, 22), and based on preclinical findings, it seems to potentially constitute a drug-sensitizing allele in the setting of MET TKI therapy (8–10). Consequently, several small molecule inhibitors of MET kinase have been developed and are currently being evaluated in clinical trials (23).

To anticipate mechanisms of acquired resistance to MET TKI therapy in the setting of *MET*-amplified NSCLC, we modeled acquired resistance to a selective MET TKI in *MET*-amplified TKI-sensitive NSCLC-derived cell lines. Through this analysis, we determined that the engagement of EGFR signaling seems to be a common mechanism by which these cells develop drug resistance. In the majority of tested drug-resistant clones, we observed either a complete switch from MET to EGFR dependency or the acquisition of codependency on these two kinases for sustained cell survival. Analysis of downstream signaling pathways in these cells suggests that the coupling of RTK signals to effectors, including AKT and ERK1/2, is a key determinant of sensitivity to these RTK inhibitors—apparently

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reflecting an "addiction" to these signaling pathways. These observations further reinforce previous findings highlighting an intimate relationship between EGFR and MET, particularly in the setting of acquired resistance to EGFR TKI treatment in NSCLC, where *MET* amplification constitutes an important mechanism (16). Considered together with those findings, our studies implicate a balance of partially redundant EGFR- and MET-transduced signals in the survival of a subset of NSCLCs and highlight the potential importance of combining MET and EGFR TKIs as a front-line therapy in this setting to prevent or delay the development of drug resistance. Our findings indicating that this combination treatment, in *EGFR* mutant or *MET*-amplified NSCLC-derived cell lines, potently suppresses the emergence of clones showing TKI resistance supports the potential clinical utility of this therapeutic strategy.

The mechanism by which EGFR signaling becomes engaged during the acquisition of MET TKI resistance in the cell line model we have examined seems to involve elevated expression of the EGFR ligand amphiregulin. By analogy, supplementary HGF, the ligand for MET, can confer resistance to EGFR TKI treatment of *EGFR* mutant NSCLCs, a finding similarly shown in a recent report, and increased HGF levels in tumors were associated with acquired EGFR TKI resistance in NSCLC patients (19). Moreover, another recent report showed that supplementary EGF can promote resistance to MET TKI treatment in *MET*-amplified gastric cancers (24), suggesting that the acquisition of resistance to MET TKIs may similarly involve a switch to EGFR signaling dependency in both *MET*-amplified NSCLCs and gastric cancers. Together, these findings highlight the potentially broader role for changes in the levels of RTK ligands in the context of acquired drug resistance.

Recent studies showed that the activated MET RTK can transactivate EGFR and/or the EGFR-related receptor ERBB3 via receptor cross-talk in *MET*-amplified NSCLC cells (8, 16, 25). Together with other studies showing cross-talk between these RTKs both in the context of normal biology and cancer (26), such findings point to an intimate relationship between the MET and EGFR pathways. Our observation that cells with acquired resistance to MET TKIs can exhibit either a switch from MET to EGFR dependency or the acquisition of EGFR/MET codependency points to an additional level of complexity in this relationship, and the mechanistic distinction between these signaling states in the various TKI-resistant clones we have generated remains unclear.

Our results suggest that prior knowledge of a predominant drug resistance mechanism can potentially be exploited to develop a combination treatment strategy that dramatically reduces the frequency with which drug resistance develops. Prompted by the discovery of *MET* gene amplification as a mechanism of acquired resistance to EGFR TKIs in *EGFR* mutant NSCLC, oncologists are already examining the potential clinical benefit of combining EGFR and MET TKIs to treat such patients. Our findings suggest that a similar combination strategy could benefit NSCLC patients showing *MET* amplification at initial diagnosis. However, we did find one NSCLC line showing *MET* gene amplification associated with high levels of phosphorylated MET but exhibiting resistance to MET TKI treatment as well as combined MET and EGFR TKI treatment. Therefore, *MET*-amplified NSCLCs may not be expected to uniformly respond to such treatments. Our observation that a subset of NSCLC-derived cell lines exhibits MET/EGFR codependency in the absence of any apparent genomic activation of these RTKs also highlights the potential significance of the EGFR-MET relationship in the context of first-line therapy strategies with EGFR and MET TKIs.

Disclosure of Potential Conflicts of Interest

J.G. Christensen: employee and shareholder, Pfizer. The other authors disclosed no potential conflicts of interest.

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