

ABSTRACT

EGF receptor (EGFR)-mutant lung cancers eventually become resistant to treatment with EGFR tyrosine kinase inhibitors (TKI). The combination of EGFR-TKI afatinib and anti-EGFR antibody cetuximab can overcome acquired resistance in mouse models and human patients. Because afatinib is also a potent HER2 inhibitor, we investigated the role of HER2 in *EGFR*-mutant tumor cells. We show *in vitro* and *in vivo* that afatinib plus cetuximab significantly inhibits HER2 phosphorylation. HER2 overexpression or knockdown confers resistance or sensitivity, respectively, in all studied cell line models. FISH analysis revealed that *HER2* was amplified in 12% of tumors with acquired resistance versus only 1% of untreated lung adenocarcinomas. Notably, *HER2* amplification and *EGFR*^{T790M} were mutually exclusive. Collectively, these results reveal a previously unrecognized mechanism of resistance to EGFR-TKIs and provide a rationale to assess the status and possibly target HER2 in *EGFR*-mutant tumors with acquired resistance to EGFR-TKIs.

SIGNIFICANCE: Because all *EGFR*-mutant lung adenocarcinomas eventually develop resistance to TKI therapy, understanding mechanisms of acquired resistance may improve clinical outcomes. These results implicate HER2 as a novel protein involved in the sensitivity or resistance of *EGFR*-mutant lung cancer and provide a rationale to assess the status of and possibly target HER2 in such tumors. *Cancer Discov*; 2(10); 922-33. ©2012 AACR.

INTRODUCTION

About 10% to 30% of tumors from patients with non-small cell lung cancer (NSCLC) harbor somatic activating mutations in the gene encoding the EGF receptor (EGFR; refs. 1-3). Tumors with the most common alterations, exon 19 deletions and exon 21 point mutations (L858R), are initially responsive to EGFR tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib (4, 5) but eventually acquire resistance. Upon disease progression, more than half of the cases harbor a second-site mutation in *EGFR*, T790M, which alters binding of drug to the ATP-binding pocket (6-8). The optimum treatment for patients with acquired resistance remains unclear.

Afatinib (BIBW2992) is a selective and potent irreversible inhibitor of EGFR and the related ERBB-family member, HER2, with IC₅₀ values of 0.5 nmol/L and 14 nmol/L, respectively (9). We previously showed in preclinical models that dual inhibition of mutant EGFR with afatinib and the anti-EGFR monoclonal antibody, cetuximab, could overcome T790M-mediated resistance (10). The combination depleted

levels of both phosphorylated and total EGFR. The role of HER2 was not investigated. The promising preclinical data led to an ongoing phase IB/II trial with highly encouraging results. Eighteen confirmed partial radiographic responses were observed in the first 45 evaluable patients with acquired resistance, leading to a 40% response rate [RR; 95% confidence interval (CI), 0.23-0.50; ref. 11]. In contrast, erlotinib plus cetuximab or afatinib alone in a similar cohort of patients showed 0% and 7% RRs, respectively (12, 13), suggesting that the combination of afatinib and cetuximab was synergistic.

Here, given the effect of afatinib on HER2, we investigated the role of HER2 in mediating the sensitivity of *EGFR*-mutant tumor cells to EGFR TKIs. We uncovered *HER2* amplification as an unrecognized mechanism of acquired resistance that occurs in a subset of tumors lacking the *EGFR*^{T790M} mutation.

RESULTS

Effect of Afatinib and Cetuximab on HER2 in Models of Acquired Resistance to Erlotinib

In previous studies of the combination of afatinib and cetuximab, we used transgenic mouse lung tumors and H1975 NSCLC cell line xenografts. In both of these models, the TKI-resistant T790M mutation was present *de novo* in *cis* with a drug-sensitive *EGFR* mutation (10). Here, we used PC9/BRC1 cells that recapitulate the acquisition of resistance; they were clonally derived from drug-sensitive PC-9 cells (exon 19 deletion) and acquired a secondary T790M mutation by long-term passage in culture (14). Consistent with our prior studies, the combination of afatinib and cetuximab in PC9/BRC1 xenografts led to greater growth inhibition than either drug did alone (Fig. 1A).

To model treatment *in vitro*, we examined the effects of the anti-EGFR agents on cell growth in 3-dimensional colony formation assays. As expected for T790M-harboring cells, erlotinib had minimal effect on the growth of PC9/BRC1

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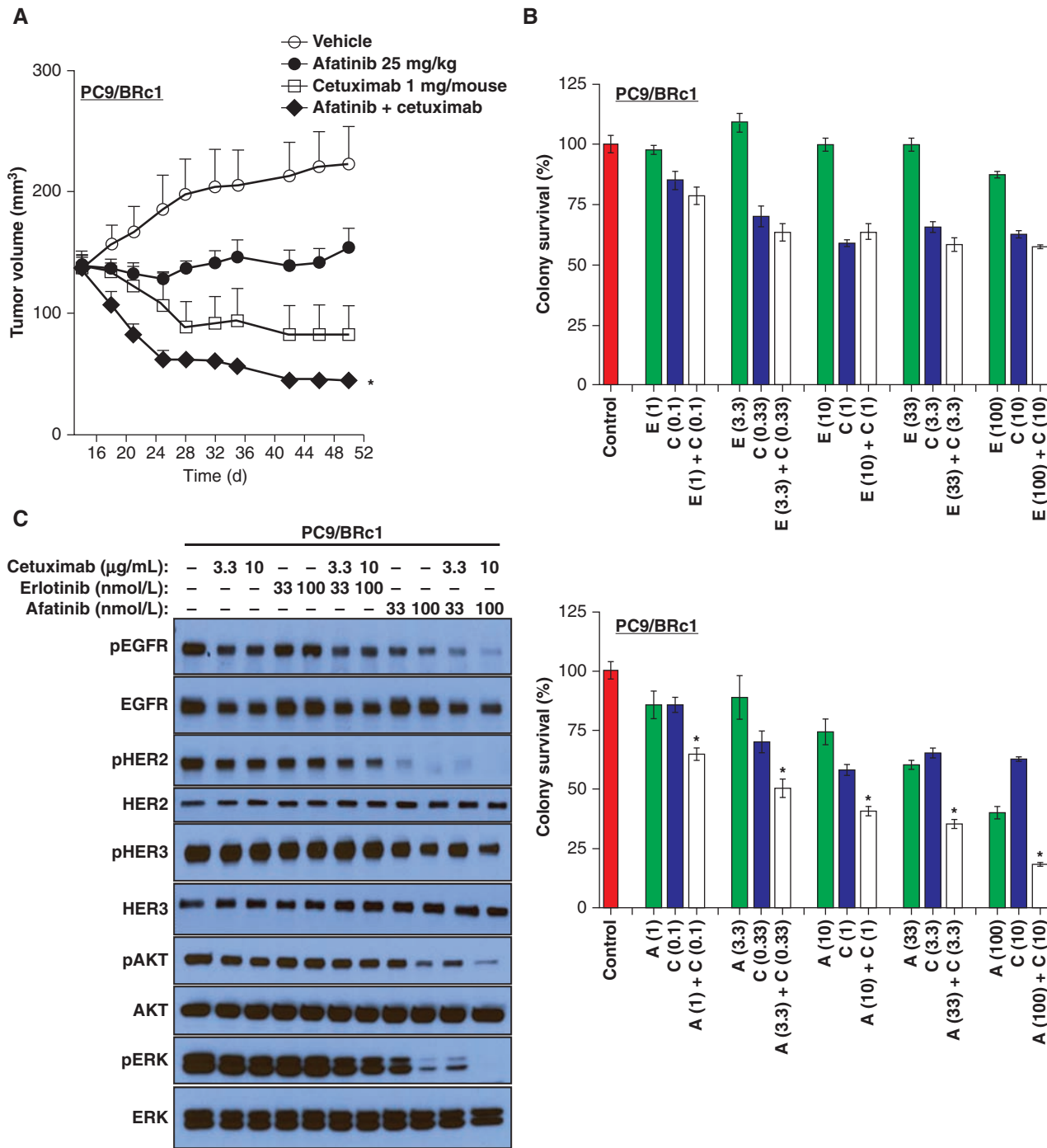


Figure 1. Effects of combination therapy with afatinib and cetuximab in *in vitro* and *in vivo* models of acquired resistance. **A**, athymic nude mice with PC9/BRC1 tumors were given vehicle, afatinib, cetuximab, or afatinib plus cetuximab. Tumor volume was determined at the indicated times after the onset of treatment. Points, values from 5 mice per group; bars, SE. *, $P < 0.05$, for the combination of afatinib plus cetuximab versus either afatinib or cetuximab alone. **B**, PC9/BRC1 cells were plated in soft agar and treated with erlotinib (E), cetuximab (C), afatinib (A), or either combination of erlotinib plus cetuximab or afatinib plus cetuximab for 8 days, after which the absorbance was measured according to the manufacturer's protocol. Data are means \pm SD of triplicates from an experiment that was repeated 3 times with similar results. *, $P < 0.05$, for the combination of afatinib plus cetuximab versus either afatinib or cetuximab alone at the same dosage. **C**, cells were serum-starved for 12 hours before treatment with the indicated drugs for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

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colonies (Fig. 1B, top). Cetuximab modestly inhibited the growth of these cells in a dose-dependent manner, but erlotinib had no additive effect (Fig. 1B, top). In contrast, the combination of afatinib and cetuximab together inhibited the growth of PC9/BRC1 cells to a significantly greater extent compared with either drug alone (Fig. 1B, bottom). Thus, results from the colony formation assays were reflective of data derived from *in vivo* xenograft models.

We next used immunoblotting studies to examine the effects of various anti-EGFR agents in PC9/BRC1 cells on levels of phosphorylated EGFR, HER2, HER3, and downstream signaling molecules, AKT and extracellular signal-regulated kinase (ERK). After 8 hours, cetuximab alone, erlotinib alone, or the combination each minimally inhibited phosphorylated levels of these proteins (Fig. 1C). In contrast, the combination of afatinib plus cetuximab significantly decreased phosphorylated levels of all of the signaling molecules (Fig. 1C).

Interestingly, afatinib alone inhibited levels of phosphorylated HER2 to a greater extent than EGFR or HER3.

Similar results were obtained using a separate resistant clone, PC9/BRC4 cells, which harbors the T790M mutation (Supplementary Fig. S1A and S1B). Comparable outcomes were also derived from other EGFR-mutant lines with T790M-mediated acquired resistance, that is, H3255/XLR and HCC827/R1 cells (ref. 14; Supplementary Fig. S1A and S1B). Incidentally, we noted that PC9/BRC1 cells express total HER2 at a higher level than parental PC9 cells upon 12-hour serum starvation (Supplementary Fig. S1C).

We further examined the status of EGFR signaling pathway proteins *in vivo* after treatment with the combination of afatinib and cetuximab for varying amounts of time. In tumor lysates derived from PC9/BRC1 xenografts, dual inhibition for 8 hours depleted levels of both phospho-EGFR and total EGFR, as previously reported (ref. 10; Fig. 2A). The effect of treatment on levels of total EGFR was greater *in vivo* than *in vitro* (Figs. 2A vs. 1C). Levels of phospho-HER2 and -HER3 were also diminished but became reactivated after 48 hours of treatment (Fig. 2A).

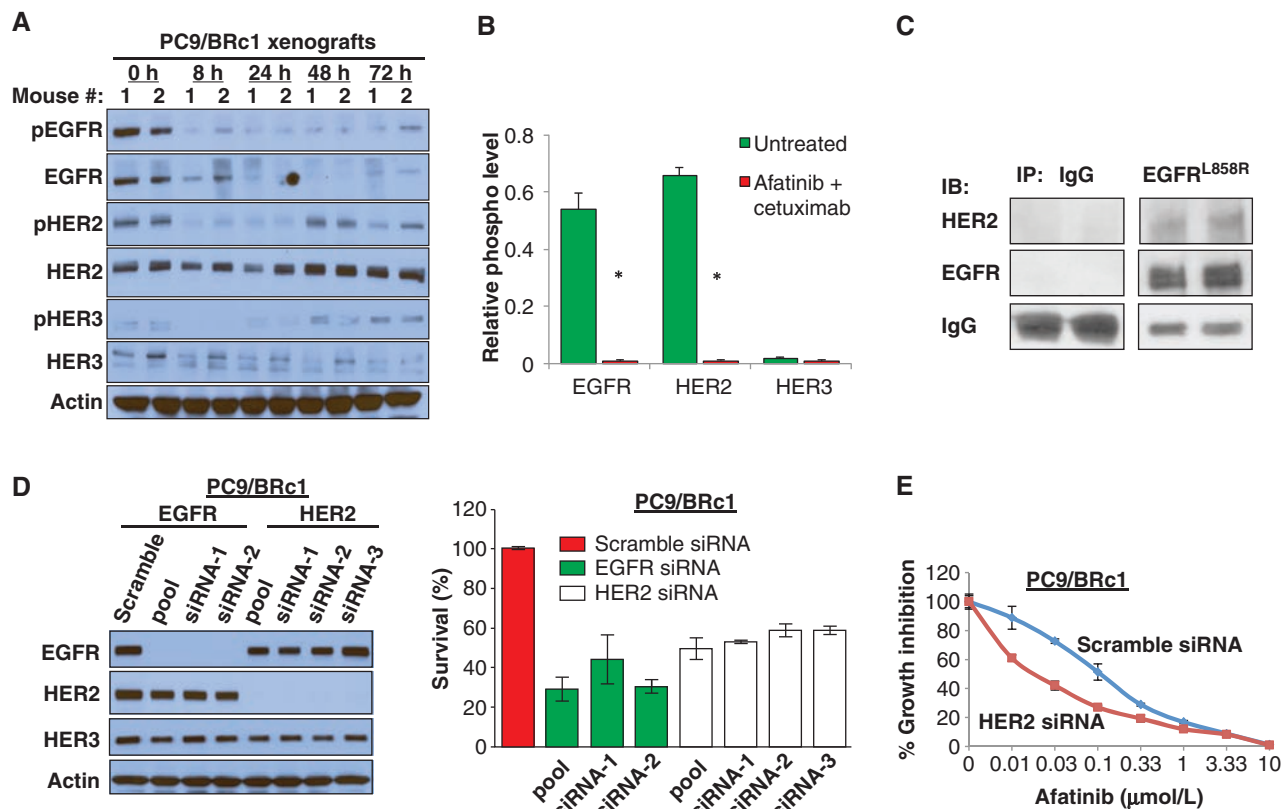


Figure 2. Role of HER2 in mediating acquired resistance to EGFR inhibition. **A**, tumor lysates from PC9/BRC1 xenograft models treated for the indicated times with the combination of afatinib plus cetuximab were subjected to immunoblot (IB) analyses with antibodies against the indicated proteins. **B**, tumor lysate (200 μg) from *CCSP-rtTA/EGFR^{L858R+T790M}* (C/L+T) mice treated with afatinib/cetuximab for 5 days, and samples from untreated controls were hybridized to phospho-RTK arrays (R&D Systems, ARY-014) in accordance with the manufacturer's protocol. Phosphorylated levels of EGFR, HER2, and HER3 were quantified using Protein Array Analyzer for ImageJ and normalized to positive control signals on the arrays. Data are presented as mean ± SEM (n ≥ 3). *, P < 0.05 for treatment in the combination of afatinib plus cetuximab versus untreated. **C**, co-immunoprecipitation of HER2 and mutant EGFR^{L858R+T790M} in transgenic mouse lung tumors driven by mutant EGFR. Lanes correspond to results from 2 individual tumors. IgG was used as immunoprecipitation (IP) control. **D**, PC9/BRC1 cells were transfected with siRNAs (scramble, EGFR siRNA sequences 1–2, or HER2 siRNA sequences 1–3) for 120 hours, after which cells were harvested and subjected to immunoblot analysis with antibodies against the indicated proteins (left), or cell viability was assessed as described in Methods (right). Data are expressed as a percentage of the value for cells transfected with scramble siRNA and are means of triplicates from experiments that were repeated 3 times with similar results. **E**, PC9/BRC1 cells were transfected with scramble or HER2 siRNAs for 24 hours, after which cells were harvested and incubated in 96-well plates for 24 hours before treatment with afatinib for 72 hours. Points represent the mean of triplicates from experiments that were repeated 3 times with similar results; bars, SD.

Similar results were obtained using transgenic animals that express human *EGFR*^{L858R+T790M} in lung epithelia (10). Here, tumor lysates from animals treated with afatinib/cetuximab for 5 days displayed lower levels of phospho-EGFR, -Her2, and tyrosine-phosphorylated protein in general than untreated controls (Fig. 2B; Supplementary Fig. S2A and S2B). The difference in Her3 phosphorylation upon treatment was not as pronounced (Fig. 2B; Supplementary Fig. S2A). Immunoprecipitation studies using an L858R-specific antibody in tumor lysates showed that Her2 coprecipitated with mutant EGFR (Fig. 2C), suggesting that these family members heterodimerize in L858R + T790M-driven mouse lung tumors.

Finally, to characterize a functional role for HER2 in resistant cells, we determined the effect of decreased HER2 expression on survival and drug sensitivity of PC9/BRC1 cells (Fig. 2D and E). Knockdown of HER2 using 2 different siRNAs led to decreased survival compared with controls, but the decrease was not as extensive as that seen upon knockdown of EGFR (Fig. 2D). Similar results were obtained with other TKI-resistant lines (Supplementary Fig. S2C). Knockdown of HER2 also increased the sensitivity of PC9/BRC1 cells to afatinib (Fig. 2E). Taken together, these data suggest that inhibition of HER2 may play an important role in the efficacy of afatinib and cetuximab in TKI-resistant *EGFR*-mutant lung adenocarcinomas.

HER2 and Sensitivity of EGFR-TKI-Resistant Cells to Panitumumab plus Afatinib

Cetuximab is a human murine chimeric antibody of the IgG1 isotype approved for use in colorectal and head and neck cancers. In humans, it may activate the complement pathway and mediate antibody-dependent cellular cytotoxicity (ADCC). Panitumumab is a related but fully humanized anti-EGFR antibody of the IgG2a subtype that may not induce complement pathway activation or ADCC (15); it is approved by the U.S. Food and Drug Administration for the treatment of colorectal cancers. To determine whether panitumumab could substitute for cetuximab, we treated PC9/BR polyclonal cells *in vivo* and *in vitro* with afatinib, panitumumab, or the combination. We obtained similar results to those with cetuximab in xenografts (Fig. 3A) and in soft agar assays (Fig. 3B). The drug combination was also highly effective at eradicating T790M-driven lung tumors in *EGFR*^{L858R+T790M} transgenic mice (Fig. 3C). Immunoblot analysis of cells treated *in vitro* revealed that the combination of afatinib and panitumumab inhibited both EGFR and HER2 phosphorylation and downregulated EGFR expression, which was similar to the results with the combination of afatinib with cetuximab (Fig. 3D). Collectively, these data show that unique anti-EGFR antibodies with different isotypes can synergize with afatinib to overcome T790M-mediated resistance and that inhibition of HER2 may play an important role in the efficacy of the combination.

Amplification of HER2 Occurs in EGFR-Mutant Tumors with Acquired Resistance to EGFR-TKIs

To explore whether Her2 levels are altered in erlotinib-resistant lung tumors, we carried out real-time PCR (RT-PCR) on lung tumor samples and adjacent normal lung from

EGFR^{L858R} mice in which long-term erlotinib treatment gave rise to resistant tumors, as previously described (16). Seven of 19 (37%) erlotinib-resistant tumors showed more than 2-fold increase in *Her2* expression compared with normal lung. Only 1 tumor also harbored the T790M mutation, whereas 3 other T790M-positive tumors had *Her2* expression levels comparable with adjacent lung tissue (Supplementary Fig. S3).

To assess the potential clinical relevance of HER2 status on lung adenocarcinomas with *EGFR* mutations, we conducted standard FISH analysis for *HER2* on tumor samples from patients with acquired resistance to gefitinib or erlotinib (Fig. 4A). *HER2* amplification was observed in 3 of 26 samples (12%). The *HER2:CEP17* ratios were 2.2, 2.7, and 5.9, respectively. These specimens also showed strong HER2 positivity by immunohistochemical staining. Notably, all 3 cases were *EGFR*^{T790M}-negative. We also conducted Sequenom testing for major and minor mutations in *KRAS* and *PIK3CA*; results were negative for all tested mutations in all samples. Histologic examination revealed no evidence of morphologic changes such as epithelial-mesenchymal transition or small-cell lung cancer, which have been observed in some cases of acquired resistance (17, 18). Given that 17 of 26 samples analyzed were *EGFR*^{T790M}-positive, *HER2* amplification was exclusive of the *EGFR*^{T790M} mutation (Fisher exact test, $P = 0.02$; Table 1). Unfortunately, baseline untreated samples were not available in the 3 patients with T790M-negative, *HER2*-amplified tumors to determine whether *HER2* amplification existed pretreatment. We further reexamined array comparative genomic hybridization data from 12 patients with acquired resistance (19) and found that 1 sample (8%), which was *EGFR*^{T790M}-negative, had a focal amplicon at 17q21, consistent with *HER2* amplification (Fig. 4B). This sample was also negative for amplification of the gene encoding the MET tyrosine kinase, which constitutes another mechanism of acquired resistance (20). Finally, we conducted *HER2* FISH on 99 untreated lung adenocarcinomas. Only 1 tumor (1%) displayed *HER2* amplification (*HER2:CEP17* ratio, 2.9; notably this tumor also had an *EGFR* mutation; 3 of 26 vs. 1 of 99; Fisher exact test, $P = 0.03$). Collectively, these results show that *HER2* amplification is detected in a subset of EGFR-TKI-resistant lung cancers; in human lung tumors, such amplification occurs in the absence of *EGFR*^{T790M}.

HER2 Contributes to the Antitumor Effects of EGFR-TKIs in Drug-Sensitive EGFR-Mutant Lung Adenocarcinoma Cells

Having implicated HER2 as a potential mediator of resistance in EGFR-TKI-resistant cells, we investigated the role of HER2 in mediating the sensitivity of parental *EGFR*-mutant cells to erlotinib. We used PC9 (exon 19 deletion), HCC827 (exon 19 deletion), and H3255 (L858R) cells, none of which harbor *EGFR*^{T790M}. Compared with controls, cells treated with increasing concentrations of erlotinib displayed reduced levels of phosphorylated HER2 as well as HER3, AKT, and ERK (Fig. 5A). Depletion of HER2 expression with siRNAs furthermore inhibited the growth of all 3 *EGFR*-mutant cell lines. However, the degree of growth inhibition was less than that observed with knockdown of EGFR itself (Fig. 5B; Supplementary Fig. S4A) or with that observed in cells more dependent upon HER2, such as BT474 breast cancer cells

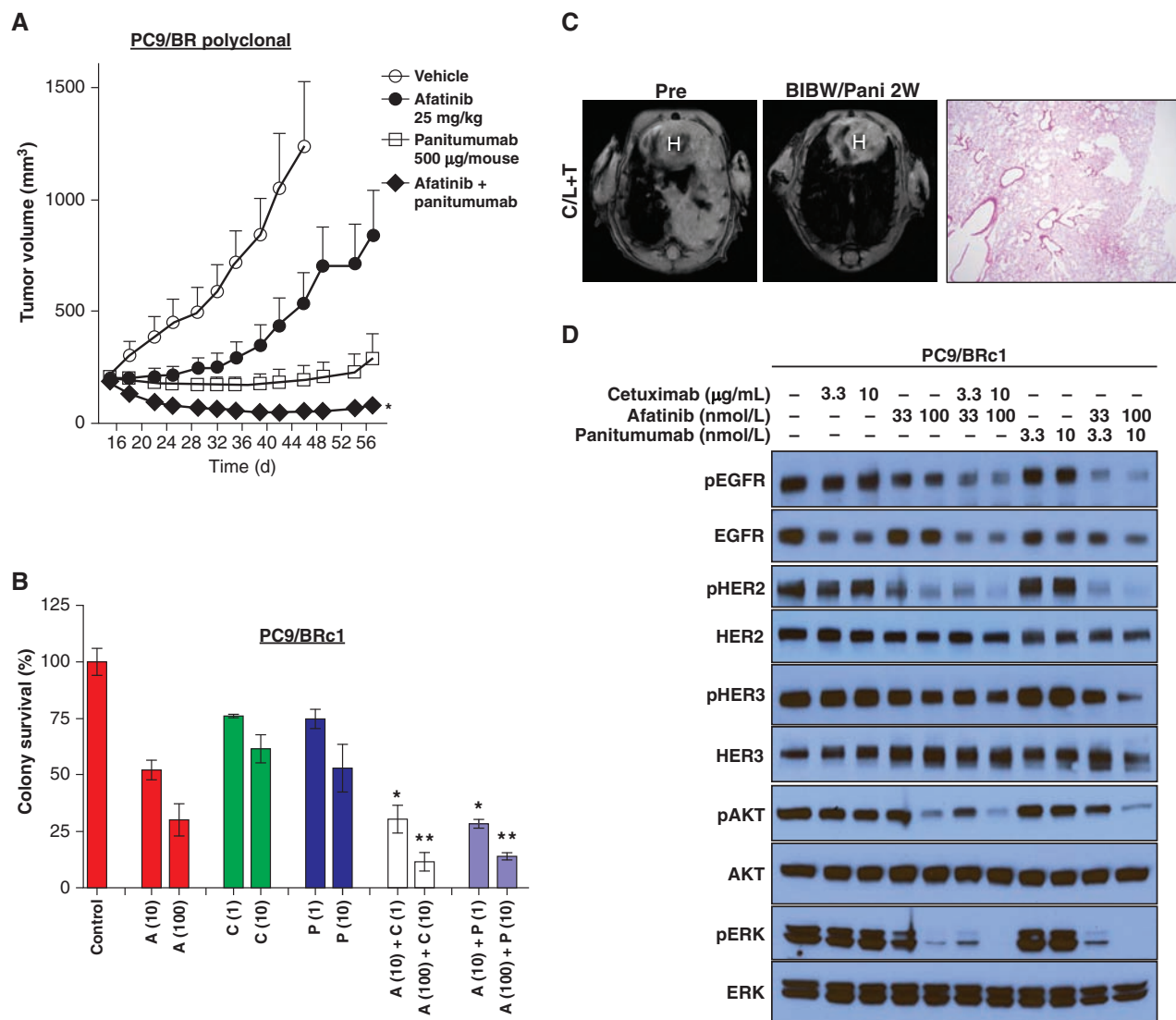


Figure 3. The effects of combined therapy with afatinib and panitumumab in *EGFR*-mutant mouse models and *in vitro*. **A**, athymic nude mice with PC9/BR polyclonal tumors were given vehicle, afatinib, panitumumab, or afatinib plus panitumumab. Points represent the values from 5 mice per group; bars, SE. $^*P < 0.05$, for the combination of afatinib plus panitumumab versus either afatinib or panitumumab alone. **B**, PC9/BRc1 cells were plated in soft agar and treated with afatinib (B), cetuximab (C), panitumumab (P), or either a combination of afatinib plus cetuximab or afatinib plus panitumumab for 8 days, after which absorbance levels were measured. Data are means with or without SD of triplicates from an experiment that was repeated 3 times with similar results. $^*P < 0.05$, for the combination of afatinib plus cetuximab versus either afatinib (10) or cetuximab (1) alone; $^{**}P < 0.05$, for the combination of afatinib plus cetuximab versus either afatinib (100) or cetuximab (10) alone. **C**, MRI images of lungs from a tumor-bearing C/L+T mouse pretreatment and after treatment with the combination of afatinib (BIBW) plus panitumumab (pani) for 2 weeks (2W). Hematoxylin and eosin–stained section from a treated C/L+T mouse (right). H, heart. **D**, cells were serum-starved for 12 hours before treatment with the indicated drugs for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies against the indicated proteins.

(Supplementary Fig. S4B). Collectively, these data show that HER2 affects the sensitivity of *EGFR*-mutant lung adenocarcinoma cells to *EGFR*-TKIs.

Finally, to confirm that HER2 overexpression causes resistance to erlotinib in *EGFR*-mutant lung adenocarcinomas, we introduced wild-type *HER2* cDNAs into *EGFR*-TKI-sensitive PC9 cells (Fig. 5C) and conducted standard growth inhibition assays (Fig. 5D and E). HER2 overexpression (>50-fold above baseline, as per densitometry assessment) conferred resistance to erlotinib (Fig. 5D) but not afatinib (Fig. 5E). Similar results were seen when *HER2* cDNAs were expressed in HCC827 cells (Supplementary Fig. S5A and S5B). Compared with control

(mock-infected) cells, erlotinib failed to inhibit phosphorylation of HER2, AKT, and ERK in PC9/*HER2* clones (Fig. 5F and G). We did not introduce *HER2* into cells harboring the T790M mutation, as the data from our clinical samples showed that these events were mutually exclusive. Collectively, these data indicate that HER2 overexpression can mediate resistance to *EGFR*-TKIs in *EGFR*-mutant NSCLCs.

DISCUSSION

All patients with metastatic *EGFR*-mutant lung cancer ultimately develop resistance to *EGFR*-TKIs gefitinib and

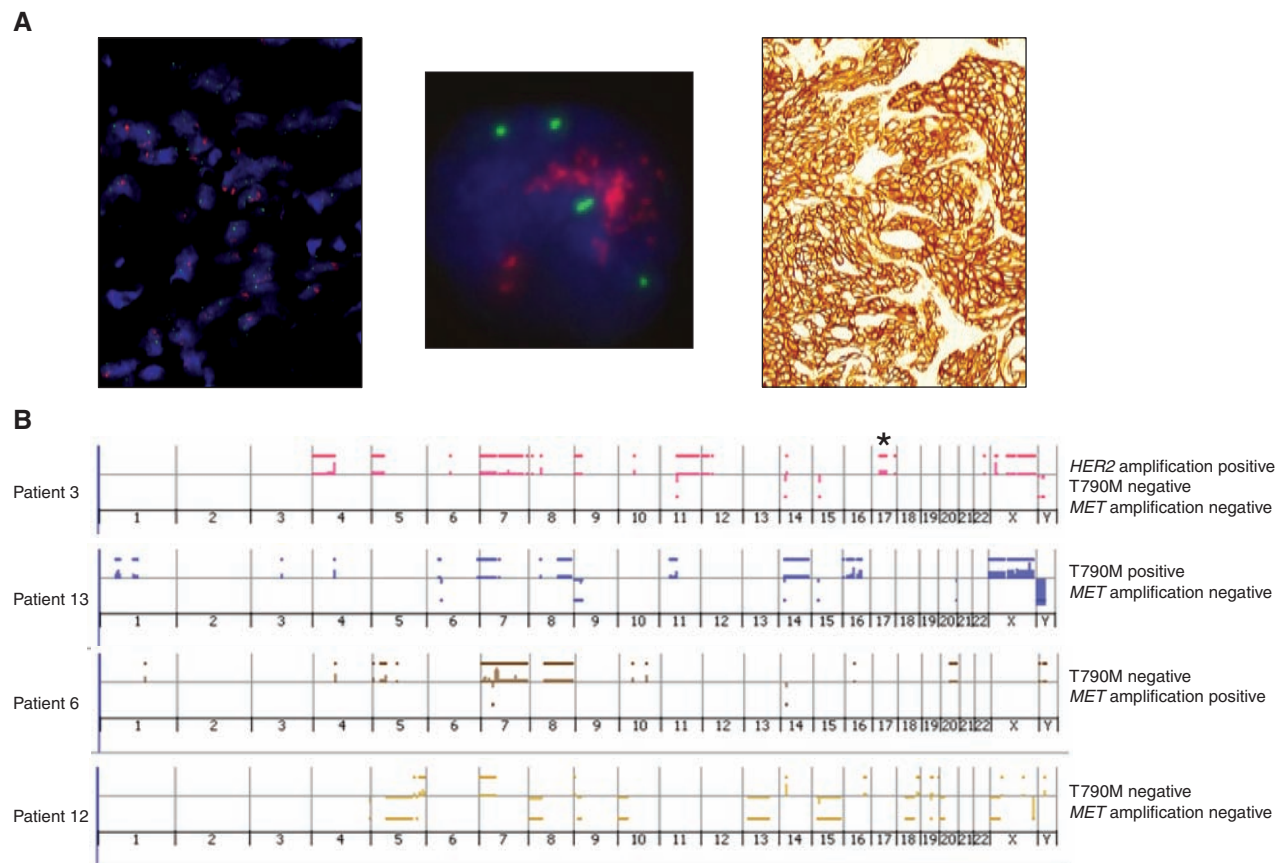


Figure 4. HER2 amplification in $EGFR^{T790M}$ -negative tumors with acquired resistance. **A**, HER2 FISH [left (low magnification), middle (high magnification)] from a tumor specimen with acquired resistance to erlotinib. HER2 (red) and CEP17 (green). The PathVysion HER2 probe kit (Abbott) was used for HER2 FISH analysis. The tumor nuclei show multiple clustered HER2 signals (red) and 2-4 chromosome 17 centromere signals (green), indicating high level HER2 amplification in this case (ratio of HER2/chromosome17 signals >2.2). Immunohistochemistry (right) was conducted on the same case using the HercepTest kit (DAKO), showing strong positive staining for HER2 (3+), according to standard scoring criteria used in breast cancer. **B**, array comparative genomic hybridization data from 12 patients with acquired resistance to gefitinib or erlotinib analyzed using a 60-mer oligonucleotide array platform [Agilent (19)]. *, amplification in HER2, occurring in 1 of 12 samples. This sample was negative for both T790M mutation and MET amplification. Bottom, representative examples without HER2 amplification.

erlotinib. The most commonly observed mechanism involves acquisition of cells harboring a second-site mutation, T790M (6-8). We previously showed in clinically relevant mouse lung tumor models that the combination of afatinib plus cetuximab could overcome T790M-mediated resistance, due to depletion of both phosphorylated and total EGFR (10). This drug combination has recently shown promising efficacy in patients with $EGFR$ -mutant tumors and acquired resistance to gefitinib or erlotinib (11). Because afatinib

inhibits HER2 in addition to EGFR, here, we investigated the role of HER2 in mediating sensitivity/resistance to EGFR-TKIs in this setting.

Through the study of preclinical *in vitro* and *in vivo* models as well as human tissues, we identify a major role for HER2 in mediating sensitivity and resistance to EGFR-TKIs. First, in multiple models of acquired resistance, levels of phosphorylated HER2 decrease after treatment with either afatinib or cetuximab alone and more dramatically with the combination. The same observations were made with afatinib plus an alternative anti-EGFR antibody, panitumumab. Second, HER2 is dephosphorylated in drug-sensitive $EGFR$ -mutant cells after treatment with erlotinib. Third, HER2 overexpression or knockdown confers resistance or sensitivity, respectively, in cell line models. Fourth, HER2 is amplified in both murine and human tumors with acquired resistance to erlotinib. Notably, in human samples, $EGFR^{T790M}$ and HER2 amplification appear mutually exclusive.

Previous studies have postulated conflicting data on the role of EGFR heterodimers in mediating sensitivity to

Table 1. $EGFR^{T790M}$ and HER2 status in 26 $EGFR$ -mutant tumors from patients with acquired resistance to erlotinib or gefitinib

Gene status	HER2 amp ⁺	HER2 amp ⁻
$EGFR^{T790M+}$	0	17
$EGFR^{T790M-}$	3	6

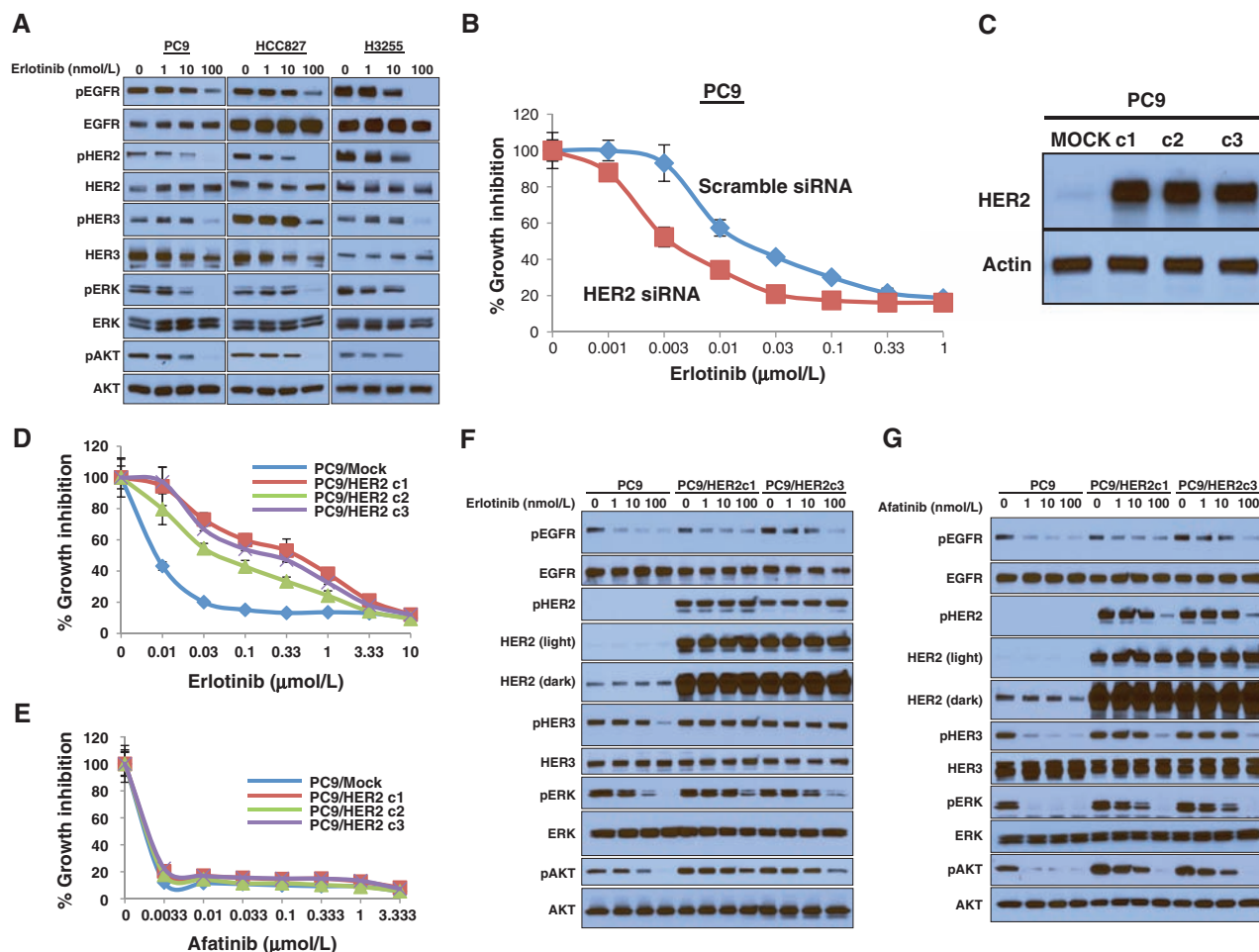


Figure 5. HER2 levels affect the sensitivity of EGFR-mutant NSCLCs to EGFR-TKIs. **A**, cells were serum-starved for 12 hours before treatment with the indicated concentrations of erlotinib for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies against the indicated proteins. **B**, PC9 cells were transfected with scramble or HER2 siRNAs for 24 hours, after which cells were harvested and incubated in 96-well plates for 24 hours before treatment with afatinib for 72 hours. Points represent mean of triplicates from experiments that were repeated 3 times with similar results; bars, SD. **C**, cells stably transfected with either empty vector (Mock) or a HER2 expression vector [clones (c) 1–3] were harvested and subjected to immunoblot analysis with antibodies to the indicated proteins. **D** and **E**, cells were incubated for 24 hours before treatment with erlotinib (**D**) or afatinib (**E**) for 72 hours, after which cell viability was assessed as described in Methods. Points, mean of triplicates from experiments that were repeated 3 times with similar results; bars, SD. **F** and **G**, cells were serum-starved for 12 hours before treatment with erlotinib (**F**) or afatinib (**G**) for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies against the indicated proteins.

EGFR-TKIs in EGFR-mutant lung cancer. Through comparison of immunoprecipitates of phosphoinositide 3-kinase (PI3K) between gefitinib-sensitive and -resistant NSCLC cell lines, a strong correlation was identified between ERBB3/HER3 expression in NSCLC cell lines and sensitivity to gefitinib, raising the possibility that ERBB3 is used to couple EGFR to the PI3K–AKT pathway in gefitinib-sensitive NSCLC cell lines harboring wild-type and mutant EGFRs (20). Alternatively, through study of receptor downregulation, data suggest that mutant EGFRs, especially the L858R/T790M variant, have a propensity to heterodimerize with HER2, which allows for evasion of CBL-mediated ubiquitinylation and subsequent lysosomal degradation (21). Our findings support the notion that HER2 influences EGFR-TKI sensitivity in EGFR-mutant cells, although we did not exclude any role for HER3. We further hypothesize that EGFR-mutant cells can become resistant either by acquiring the T790M mutation, which enhances

HER2 heterodimerization in the absence of HER2 amplification, or by acquiring HER2 amplification in the absence of a second-site mutation. Future biochemical studies and/or genetic studies using ErbB2 or ErbB3 knockouts in the context of EGFR-mutant lung cancers may help shed further light on this issue.

In the present study, we found that HER2 amplification is a rare event in untreated lung adenocarcinomas, occurring in 1% of samples as assessed by FISH. In contrast, other groups have reported that HER2 may be amplified in up to 22.8% of NSCLCs (22–25). This high figure appears inconsistent with those from other data sets. For example, microarray-based genomic copy number analyses have shown that HER2 copy number gain occurred in only 10 of 628 (1.6%) NSCLCs (26). In our own previously published genomic studies, HER2 gene copy gain occurred in only 2 of 199 (1%) lung adenocarcinomas, neither of which harbored

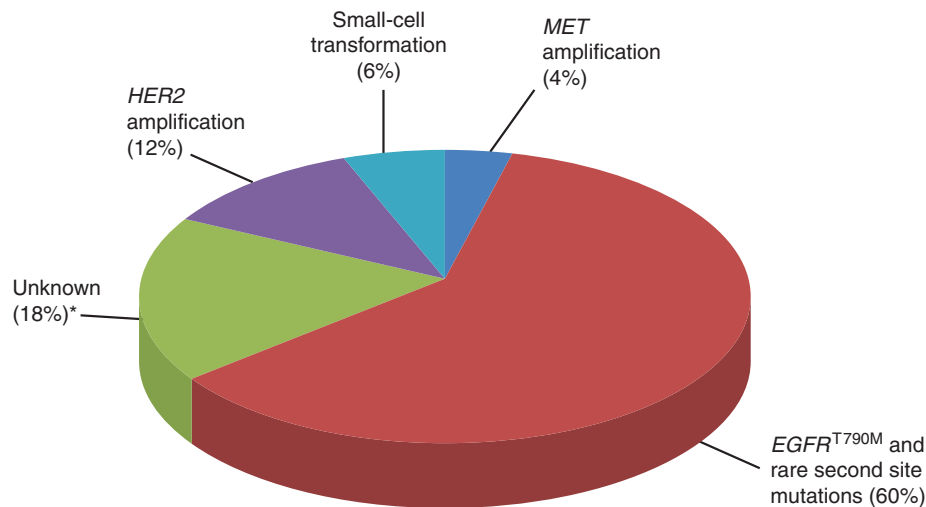


Figure 6. Frequency of mechanisms of acquired resistance to EGFR-TKIs in EGFR-mutant lung cancer. *Includes epithelial-mesenchymal transition, present at an uncertain prevalence. [Adapted from (34).]

EGFR mutations (27). Further systematic analyses of HER2 status in both untreated NSCLCs and those with acquired resistance to EGFR-TKIs are warranted.

Activation of HER2 signaling was recently reported to cause resistance to cetuximab alone in patients with colorectal cancer (28, 29). In one of the studies (29), introduction of HER2 into another EGFR drug-sensitive lung cancer cell line, HCC827, failed to affect drug sensitivity, consistent with our findings (Supplementary Fig. S5). These data suggest that the effects of HER2 expression or activity on sensitivity/resistance to anti-EGFR drugs may depend on cell line lineage or other as yet undefined genetic determinants. The studies in colon cancer suggest that the combination of afatinib plus cetuximab could also be efficacious in overcoming acquired resistance to cetuximab in that disease.

In summary, our data suggest that the ERBB family member, HER2, plays a significant role in mediating sensitivity of EGFR-mutant lung tumors to anti-EGFR therapy. We identify HER2 amplification as a new mechanism of acquired resistance to EGFR-TKIs in EGFR-mutant NSCLC tumors, occurring independently of the EGFR^{T790M} secondary mutation. This observation could explain why the combination of afatinib/cetuximab induces responses in some but not all patients without T790M-mediated acquired resistance (11). If the 12% prevalence of HER2 amplification in this clinical setting is verified in future studies, this would place it as one of the most common acquired resistance mechanisms after the EGFR^{T790M} mutation (ref. 34; Fig. 6). Hopefully, this knowledge will eventually lead to improved therapeutic outcomes for patients with EGFR-mutant lung cancer.

METHODS

Cell Culture and Derivation of TKI-Resistant Lines

EGFR-mutant PC9 cells (del E746-A750) or HCC827 cells (del E746-A750) were cultured in RPMI media (American Type Culture Collection) supplemented with 10% heat-inactivated FBS (Gemini Bio Products). These cell lines have been used as reagents in the Pao Lab since 2005 (7, 30). Cell lines were genotyped multiple

times to confirm the presence of known EGFR mutations by standard Sanger sequencing. Cells were grown in a humidified incubator with 5% CO₂ at 37°C. Resistant cells were derived as previously described (14). Briefly, parental cells were cultured with increasing concentrations of TKIs starting with the IC₅₀. Doses were increased in a stepwise pattern when normal cell proliferation patterns resumed. Fresh drug was added every 72 to 96 hours. Resistant cells were maintained initially as polyclonal populations under constant TKI selection. Clonal-resistant cells were isolated by limiting dilution.

Xenograft Studies

Nude mice (*nu/nu*; Harlan Laboratories) were used for *in vivo* studies and were cared for in accordance with guidelines approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee and Research Animal Resource Center (New York, NY). Eight-week-old female mice were injected s.c. with 10 million PC9/BRC1 cells. When tumors reached approximately 150 mm³, animals were randomized to receive vehicle alone, cetuximab (1 mg/mouse twice per week, intraperitoneally; Bristol-Myers Squibb/ImClone/Eli Lilly) or panitumumab (500 µg/mouse twice per week, intraperitoneally; Amgen), afatinib (25 mg/kg daily, orally; drug synthesized by the MSKCC Organic Synthesis Core), or a combination of both afatinib and either cetuximab or panitumumab. Achievable afatinib serum levels at this dose range from 80 to 667 nmol/L (9); the standard dose in humans is 40 mg orally once daily; at 45 mg, the C_{max} is 141 nmol/L (31). Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula: length × width² × 0.52. Body weight was also assessed twice weekly. Tumor samples were collected within 8 hours of the last treatment. Each sample was cut in halves; one half was preserved in 4% paraformaldehyde and one half was flash-frozen in liquid nitrogen and stored at -80°C until further use.

Transgenic Mouse Studies

All animals were kept in pathogen-free housing under guidelines approved by the Yale University or Vanderbilt-Ingram Cancer Center Institutional Animal Care and Use Committees. The generation of EGFR^{L858R+T790M} mice was previously described (10). Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets (625 ppm; Harlan-Teklad). Afatinib was

suspended in 0.5% (w/v) methylcellulose and injected intraperitoneally at the dose of 25 mg/kg daily for 5 days. Cetuximab was administered intraperitoneally at 1 mg/mouse twice per week. Panitumumab was administered intraperitoneally at 500 µg/mouse twice per week.

Soft Agar Assay

The colony-forming capacity of PC9/BRC1 cells was assessed using the CytoSelect 96-Well In Vitro Tumor Sensitivity Assay (Soft Agar Colony Formation) Kit (Cell Biolabs Inc.), according to the manufacturer's protocol. Briefly, 50 µL of base agar matrix layer was dispensed into each well of a 96-well tissue culture plate. Cells (5×10^3) in 75 µL of cell suspension/agar matrix layer were dispensed into each well. The cells were treated with 50 µL of culture medium containing various drugs. After incubation for 8 days, 125 µL of the $\times 1$ matrix solubilization buffer was added to solubilize the agar matrix completely, and MTT was added to each well. The absorbance was then recorded on a SpectraMax fluorometer at 570 nm.

Immunoblotting

Cells were washed with cold PBS and lysed for 30 minutes with radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40 substitute, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche), 40 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 1 µmol/L okadaic acid. Protein levels were quantified with Bradford Reagent (Bio-Rad), and equal amounts were loaded for SDS-PAGE using 4% to 20% acrylamide precast gels (Invitrogen), followed by transfer to polyvinylidene difluoride membranes. Membranes were blotted with pEGFR (Y1068, CST, catalog number 2234), total EGFR (BD Biosciences, catalog number 610016), EGFR^{L858R} (CST, catalog number 3197), pHER2 (Y1248, CST, catalog number 2247), total HER2 for mouse (Millipore, catalog number 06-562), pHER3 (Y1197, CST, catalog number 4561), total HER3 for mouse (NanoTools, catalog number 0237), pAKT (S473, CST, catalog number 9271), total AKT (CST, catalog number 9272), pERK (T202/204, catalog number 9101), total ERK (catalog number 9102), Pro-surfactant Protein C (Abcam, catalog number ab90716), and actin (Sigma-Aldrich) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies or pY-HRP (R&D Systems, part number 841403). All antibodies were purchased from Cell Signaling Technology, unless noted. Signals were detected with Western blotting detection reagents (Perkin Elmer, NEL10500NEA).

Growth Inhibition Assay

Cellular growth inhibition was measured with CellTiter Blue Reagent (Promega, G8081) according to the manufacturer's instructions using cells plated in triplicate at a density of 2,000 cells per well. Fluorescence was measured on a SpectraMax fluorometer. Growth inhibition was calculated as percentage of vehicle-treated wells \pm SD.

RNA Interference

Cells were plated at 50% to 60% confluence in 6-cm dishes and then incubated for 24 hours before transient transfection for the indicated times with siRNAs mixed with Lipofectamine reagent (Invitrogen). siRNAs specific for EGFR (MQ-003114-03) and HER2 (MQ-003126-04) mRNA were obtained from Dharmacon.

FISH Analysis

Assessment of *HER2* gene copy number was conducted on formalin-fixed, paraffin-embedded specimens. The Vysis PathVysion HER-2 DNA Probe Kit (Abbott Laboratories) was used following a standard manufacturer's protocol. At least 40 cells were analyzed for each case by 2 reviewers and were classified as amplified if the *HER2/CEP17*

ratio per cell was >2 or homogeneously staining regions with >15 copies in $>10\%$ of the cells were present. Cases with a ratio between 1.8 and 2.2 were initially considered as borderline range, prompting further review and recounting of wider areas to confirm their status as amplified or not amplified.

Cell Transfection

A pBabe-puro vector encoding wild-type HER2 was kindly provided by Carlos Arteaga (32). The pBabe-puro/WT-HER2 vector and the pVSV-G vector (Clontech) for production of the viral envelope were introduced into GP2-293 cells (80% confluence in a 10-cm dish) with the use of FuGENE6 transfection reagent. After 48 hours, viral particles released into the culture medium were concentrated by centrifugation at $15,000 \times g$ for 3 hours at 4°C. The resulting pellet was then suspended in fresh RPMI-1640 medium and used to infect PC9 and HCC827 cells as previously described (33).

Quantitative RT-PCR

Mouse lung tissue samples were crushed, and RNA was extracted from the tissue powders using TRIzol reagent (Invitrogen). RNA was DNase-treated (Invitrogen) to eliminate any contaminating DNA. cDNA was made from 600 ng of DNase-treated RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Resulting cDNA was diluted 10-fold and 2 µL was used for quantitative RT-PCR analysis using the mouse Her2 and surfactant protein C TaqMan Gene Expression Assays (Applied Biosystems). Reactions were conducted in a 10-µL volume using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and run in triplicates on the Applied Biosystems ViiA7 Real-Time PCR System.

Immunoprecipitation

Lung tissue containing tumors from mutant *EGFR*^{L858R+T790M} transgenic mice were pulverized and lysed in coimmunoprecipitation buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, and 1% NP-40 plus the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). After preclearing lysates with protein A/G plus beads, 500 µg of protein was incubated with 5 µg of antibody against *EGFR*^{L858R} overnight at 4°C. Lysates were further incubated with 40 µL of 50% protein A/G plus beads for 1 hour at 4°C, followed by 5 washes with lysis buffer. Immunoblotting was conducted as described above.

Statistical Analysis

Quantitative data for Western blotting and receptor tyrosine kinase (RTK) arrays are presented as means \pm SD and were analyzed by Student 2-tailed *t* test. A value of $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

V.A. Miller has consulted for Astellas, Boehringer-Ingelheim, Clovis, and Genentech and currently is an employee of Foundation Medicine. W. Pao has consulted for Clovis, MolecularMD, Bristol-Myers Squibb, and AstraZeneca and has research funding from Clovis, Symphogen, Bristol-Myers Squibb, and AstraZeneca. Y.Y. Janjigian has received research funding from Boehringer-Ingelheim and Roche/Genentech. G.J. Riely has consulted for Chugai, Ariad, Tragara, Daiichi, Novartis, and Abbott Molecular and has research funding from GSK, Boehringer-Ingelheim, Novartis, Chugai, Infinity, Bristol-Myers Squibb, and Pfizer. M.G. Kris has consulted for Pfizer, Boehringer Ingelheim, and Genentech/Roche and has research funding from Pfizer and Boehringer-Ingelheim. Rights to a patent application for *EGFR*^{T790M} testing were licensed on behalf of V.A. Miller, K. Politi, and W. Pao by Memorial Sloan-Kettering Cancer Center to MolecularMD. No potential conflicts of interest were disclosed by the other authors.

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