HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR mutant lung cancers that lack the second-site EGFR T790M mutation

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Abstract

EGFR-mutant lung cancers eventually become resistant to treatment with EGFR tyrosine kinase inhibitors (TKIs). The combination of EGFR-TKI afatinib and anti-EGFR antibody cetuximab can overcome acquired resistance in mouse models and human patients. Since 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. Fluorescent *in situ* hybridization 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.

Keywords

EGFR mutations; lung cancer; EGFR tyrosine kinase inhibitors; erlotinib; afatinib; cetuximab; HER2 amplification; EGFR T790M; acquired resistance

Introduction

About 10–30% of tumors from non-small cell lung cancer (NSCLC) patients harbor somatic activating mutations in the gene encoding the epidermal growth factor receptor (EGFR) (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 (TKIs) 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 and 14 nM, 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 (95% CI 0.23–0.50) (11). By contrast, erlotinib plus cetuximab or afatinib alone in a similar cohort of patients showed 0% and 7% response rates (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 utilized 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, 15). Consistent with our prior studies, the combination of afatinib and cetuximab in PC9/BRc1 xenografts led to greater growth inhibition than either drug alone (Fig. 1A).

To model treatment *in vitro* we examined the effects of the anti-EGFR agents on cell growth in three dimensional colony formation assays. As expected for T790M-harboring cells, erlotinib had minimal effect on the growth of PC9/BRc1 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**). By 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 ERK. After 8 hours, cetuximab alone, erlotinib alone, or the combination each minimally inhibited phosphorylated levels of these proteins (Fig. 1C). By 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 Figs. 1A, B). Comparable outcomes were also derived from other EGFR mutant lines with T790M-mediated acquired resistance, i.e. H3255/XLR and HCC827/R1 cells (14) (Supplementary Figs. 1A, B). 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. 1C).

We further examined the status of EGFR signaling pathway proteins *in vivo* after treatment with the combination of drugs 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 (10) (Fig. 2A). The effect of treatment on levels of total EGFR was greater *in vivo* than *in vitro* (Fig. 2A vs. Fig. 1C). Levels of phospho-HER2 and -HER3 were also diminished but became reactivated after 48 hours of treatment (Fig. 2A).

Similar results were obtained using transgenic animals that express human EGFR$^{L858R+T790M}$ in lung epithelia (16). Here, tumor lysates from animals treated with afatinib/cetuximab for five days displayed lower levels of phospho-EGFR, -Her2, and tyrosine-phosphorylated protein in general compared to untreated controls (Fig. 2B, Supplementary Figs. 2A and 2B). The difference in Her3 phosphorylation upon treatment was not as pronounced (Fig. 2B, Supplementary Fig. 2A). Immunoprecipitation studies using an L858R-specific antibody in tumor lysates showed that Her2 co-precipitated 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 (Figs. 2D and 2E). Knockdown of HER2 using two different short-interfering RNAs (siRNAs) led to decreased survival compared to controls, but the decrease was not as extensive as that seen upon knockdown of EGFR (Fig. 2D). Similar results were obtained with other EGFR-resistant lines (Supplementary Fig. 2C). 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 (16); it is approved for the treatment of colorectal cancers. To determine if 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 down-regulated EGFR expression similar to 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 performed quantitative 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 (17). Seven of nineteen (37%) erlotinib-resistant tumors showed >2-fold increase in *Her2* expression compared to normal lung. Only one tumor also harbored the T790M mutation, while three other T790M-positive tumors had Her2 expression levels comparable to adjacent lung tissue (Supplementary Figure 3).

To assess the potential clinical relevance of HER2 status on lung adenocarcinomas with *EGFR* mutations, we performed standard fluorescent *in situ* hybridization (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 three 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 has been observed in some cases of acquired resistance (18), (19). Given that 17 of 26 samples analyzed were EGFR T790M positive, *HER2* amplification was exclusive of the EGFR T790M mutation (Fisher's Exact Test, *p*=0.02, Table 1). Unfortunately, baseline untreated samples were not available in the three patients with T790M-negative, *HER2*-amplified tumors to determine if *HER2* amplification existed pre-treatment. We further re-examined array comparative genomic hybridization data from 12 patients with acquired resistance (20) and found that one 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 performed *HER2* FISH on 99 untreated lung adenocarcinomas. Only one (1%) tumor displayed *HER2* amplification (HER2: CEP17 ratio: 2.9; notably this tumor also had an *EGFR* mutation) (3/26 vs 1/99; p=0.03; Fisher's exact test). Collectively, these results demonstrate 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 to 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 three *EGFR* mutant cell lines. However, the degree of growth inhibition was less than that observed with knockdown of EGFR itself (Fig. 5B, Supplementary Fig. 4A) or with that observed in cells more dependent upon HER2, such as BT474 breast cancer cells (Supplementary Fig. 4B). Collectively, these data demonstrate 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 performed standard growth inhibition assays (Fig. 5D, 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. 5A, B). Compared to control (mock-infected) cells, erlotinib failed to inhibit phosphorylation of HER2, AKT and ERK, in PC9/HER2 clones (Fig. 5F, 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 NSCLC.

Discussion

All patients with metastatic EGFR mutant lung cancer ultimately develop resistance to EGFR-TKIs gefitinib and erlotinib. The most commonly observed mechanism involves acquisition of cells harboring a second-site mutation, T790M (6–8). We previously demonstrated 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 humans 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 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 WT and mutant EGFRs (15). Alternatively, through study of receptor down-regulation, data suggests 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. By contrast, others group have reported that *HER2* may be amplified, even up to 22.8% of NSCLCs (22–25). This high figure appears inconsistent with other datasets. 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 genomic studies, *HER2* gene copy gain occurred in only 2 of 199 (1%) lung adenocarcinomas, neither of which harbored *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. 5). 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 (30). 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 (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 (ATCC) supplemented with 10% heat inactivated fetal bovine serum (Gemini Bio Products). These lines have been used as reagents in the Pao Lab since 2005 (7), (31). Cell lines were re-genotyped multiple times to confirm the presence of known EGFR mutations using standard Sanger sequencing. Cells were grown in a humidified incubator with 5% CO_2 at 37°C. Resistant cells were derived as previously described (14). Briefly, parental cells were cultured with increasing concentrations of TKIs starting with the IC30. Doses were increased in a stepwise pattern when normal cell proliferation patterns resumed. Fresh drug was added every 72–96 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 MSKCC Institutional Animal Care and Use Committee and Research Animal Resource Center. Eight-week-old female mice were injected s.c. with 10 million PC9/BRc1 cells. When tumors reached ~150 mm^{3}animals were randomized to receive vehicle alone, cetuximab (1 mg/mouse twice per week, intraperitoneally; Bristol-Myers Squibb/ImClone/Eli Lilly) or panitumumab (500 ug/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 80–667 nM (9); the standard dose in humans is 40 mg po qd; at 45mg, the Cmax is 141 nM (32). 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 $\times$ width^2 $\times$ 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 or VICC 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 i.p. at 1 mg/mouse 2 times/week. Panitumumab was administered i.p. at 500 μg/mouse 2 times/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 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 $1 \times$ Matrix Solubilization Buffer was added to solubilize the agar matrix completely, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide 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 RIPA buffer (150mM Tris.HCl pH 7.5, 150mM NaCl, 1% NP-40 substitute, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche), 40mM sodium fluoride, 1mM sodium orthovanadate, and 1μM okadaic acid. Protein levels were quantified with Bradford Reagent (Bio-Rad) and equal amounts were loaded for SDS-PAGE using 4–20% acrylamide precast gels (Invitrogen), followed by transfer to PVDF membranes. Membranes were blotted with pEGFR (Y1068, CST, cat#2234), total EGFR (BD Biosciences, cat# 610016), EGFR^{L858R} (CST, cat# 3197), pHER2 (Y1248, CST, cat# 2247), total HER2 for mouse (Millipore, cat#06-562), pHER3 (Y1197, CST, cat# 4561), total HER3 for mouse (NanoTools,

cat#0237), pAKT (S473, CST, cat#9271), total AKT (CST, cat#9272), pERK (T202/204, cat#9101), total ERK (cat# 9102), Pro-surfactant Protein C (Abcam, cat#ab90716) and actin (Sigma-Aldrich) followed by HRP conjugated secondary antibodies or pY-HRP (R&D Systems, part#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) as per the manufacturer's instructions using cells plated in triplicate at a density of 2000 cells per well. Fluorescence was measured on a SpectraMax fluorometer. Growth inhibition was calculated as percentage of vehicle-treated wells ± 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 performed on formalin-fixed paraffin-embedded specimens. The Vysis PathVysion HER-2 DNA Probe Kit (Abbott Laboratories, Abbott Park, IL) was used following 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 (30).The pBabe-puro/WT-HER2 vector and the pVSV-G vector (Clontech, Palo Alto, CA, USA) 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 48h, viral particles released into the culture medium were concentrated by centrifugation at 15,000g for 3 h 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 600ng 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 were used for quantitative RT-PCR analysis using the mouse Her2 and surfactant protein C TaqMan Gene Expression Assays (Applied Biosystems). Reactions were performed 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 co-immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris HCl and 1% NP40 plus the Halt protease and phosphotase inhibitor cocktail (Thermo Scientific). After pre-clearing 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 performed as described above.

Statistical analysis

Quantitative data for western blot and RTK arrays are presented as means ± SD and were analyzed by Student's 2-tailed t test. A value of $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

EGFR	epidermal growth factor receptor
TKI	tyrosine kinase inhibitor
HER2/3	human epidermal growth factor receptor 2/3
NSCLC	non-small cell lung cancer
ATP	adenosine triphosphate
RR	response rate
ADCC	antibody-dependent cellular cytotoxicity
BR	BIBW2992 resistant
BRc1	BIBW2992-resistant clone 1
FISH	fluorescent *in situ* hybridization

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Statement of 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.
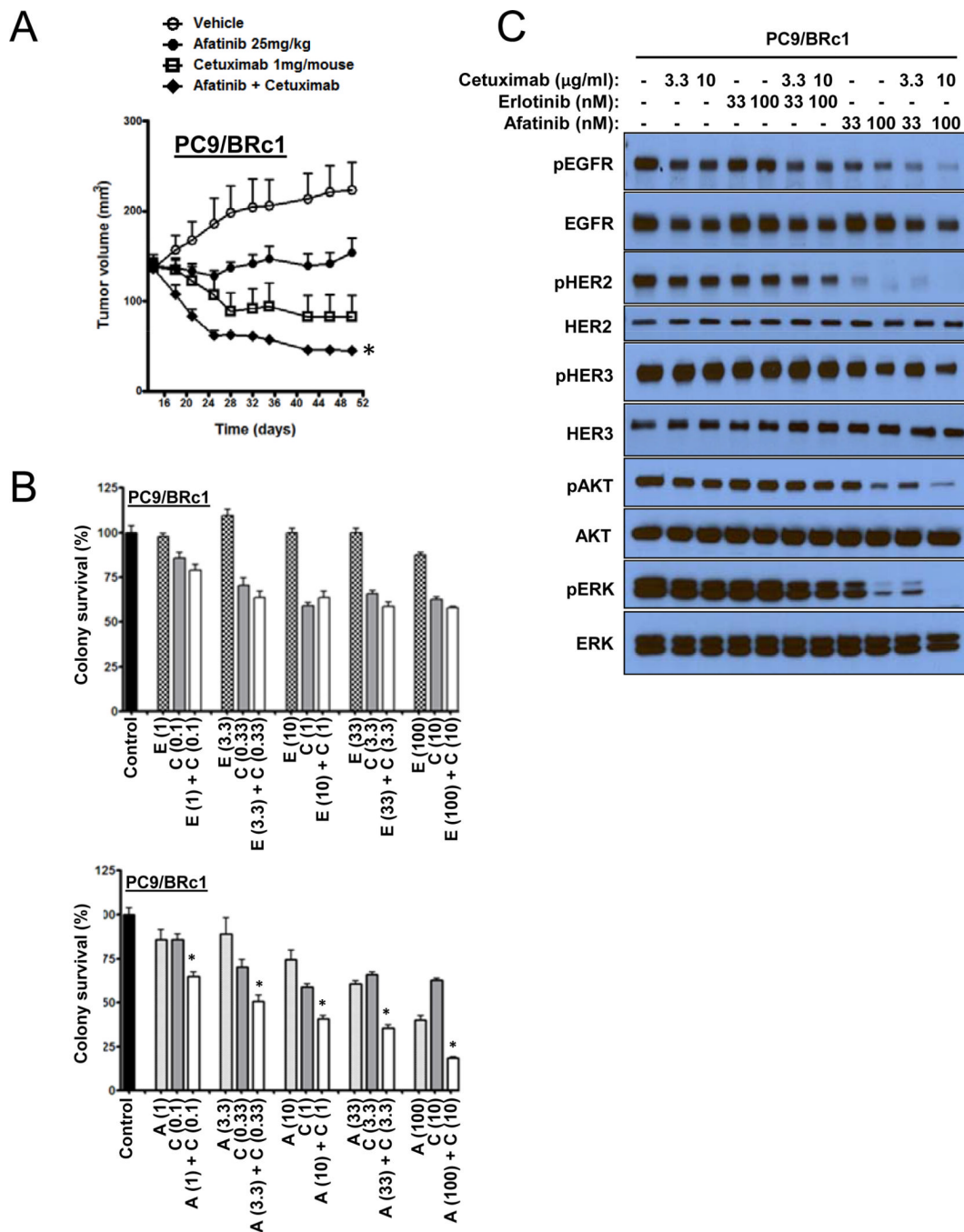
Figure 1. Effects of combination therapy with afatinib and cetuximab in *in vitro* and *in vivo* models of acquired resistance

(A) Athymic nude mice with PC9/BRc1 tumors were administered vehicle, afatinib, cetuximab, or afatinib plus cetuximab. Tumor volume was determined at the indicated times after the onset of treatment. Points, values from five 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 ± SD of triplicates from an experiment that was repeated a total of 3 times

with similar results. **(C)** Cells were serum starved for 12 hours prior to treatment with the indicated drugs for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.
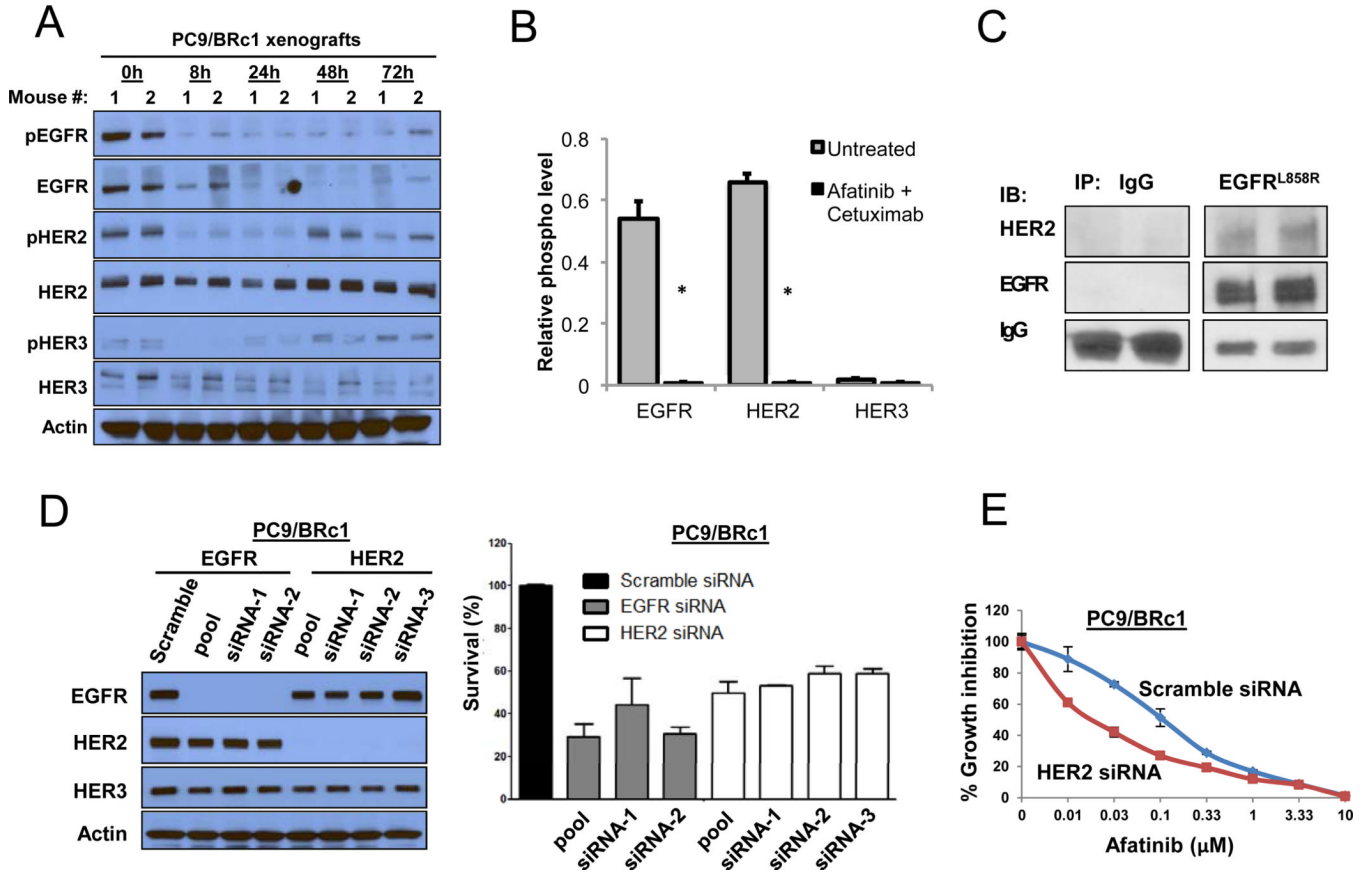
Figure 2. Role of HER2 in mediating acquired resistance to EGFR inhibition
(A) Tumor lysates from PC9/BRc1 xenograft models treated for the indicated times with the combination of afatinib plus cetuximab were subjected to immunoblot analyses with antibodies against the indicated proteins. **(B)** 200 μg of tumor lysate 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 to 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). **(C)** Co-immunopreciptiation of HER2 and mutant EGFR L858R+T790M in transgenic mouse lung tumors driven by mutant EGFR. IgG was used as immune-precipitation 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 a total of 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 prior to treatment with afatinib for 72 hours. Points, mean of triplicates from experiments that were repeated a total of three times with similar results; bars, SD.
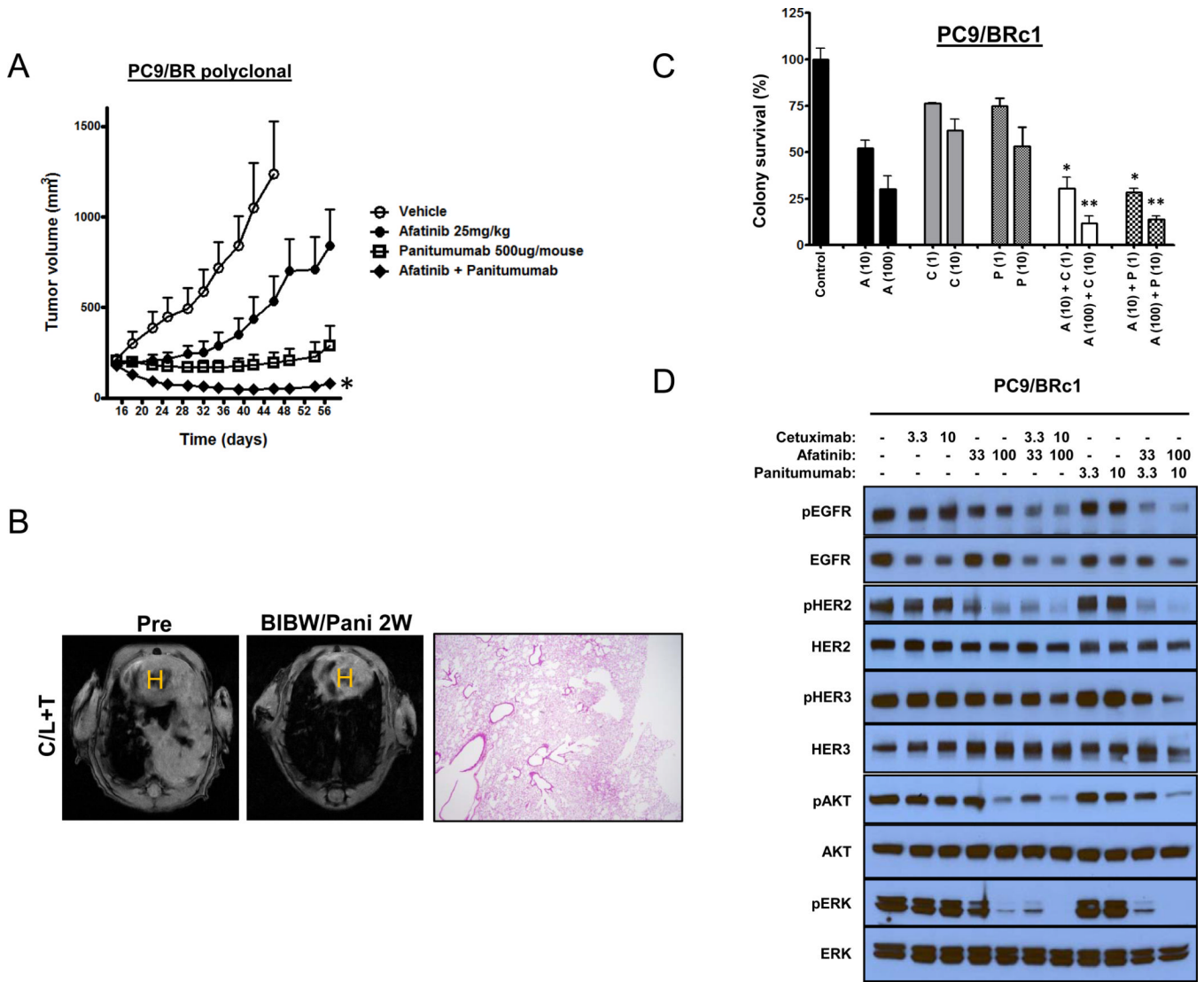
Figure 3. The effects of combined therapy with afatinib and panitumumab in EGFR-mutant mouse models and *in vitro*

(**A**) Athymic nude mice with PC9/BR polyclonal tumors were administered vehicle, afatinib, panitumumab, or afatinib plus panitumumab. Points, values from five mice per group; bars, SE. *, *P* < 0.05, for the combination of afatinib plus panitumumab versus either afatinib or panitumumab alone. (**B**) MRI images of lungs from a tumor-bearing C/L+T mouse pretreatment and after treatment with the combination of afatinib plus panitumumab (pani) for 2 weeks (2W). H&E-stained section from a treated C/L+T mouse (right panels) (**C**) 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 ± SD of triplicates from an experiment that was repeated a total of 3 times with similar results. (**D**) Cells were serum starved for 12 hours prior to treatment with the indicated drugs for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies against the indicated proteins.
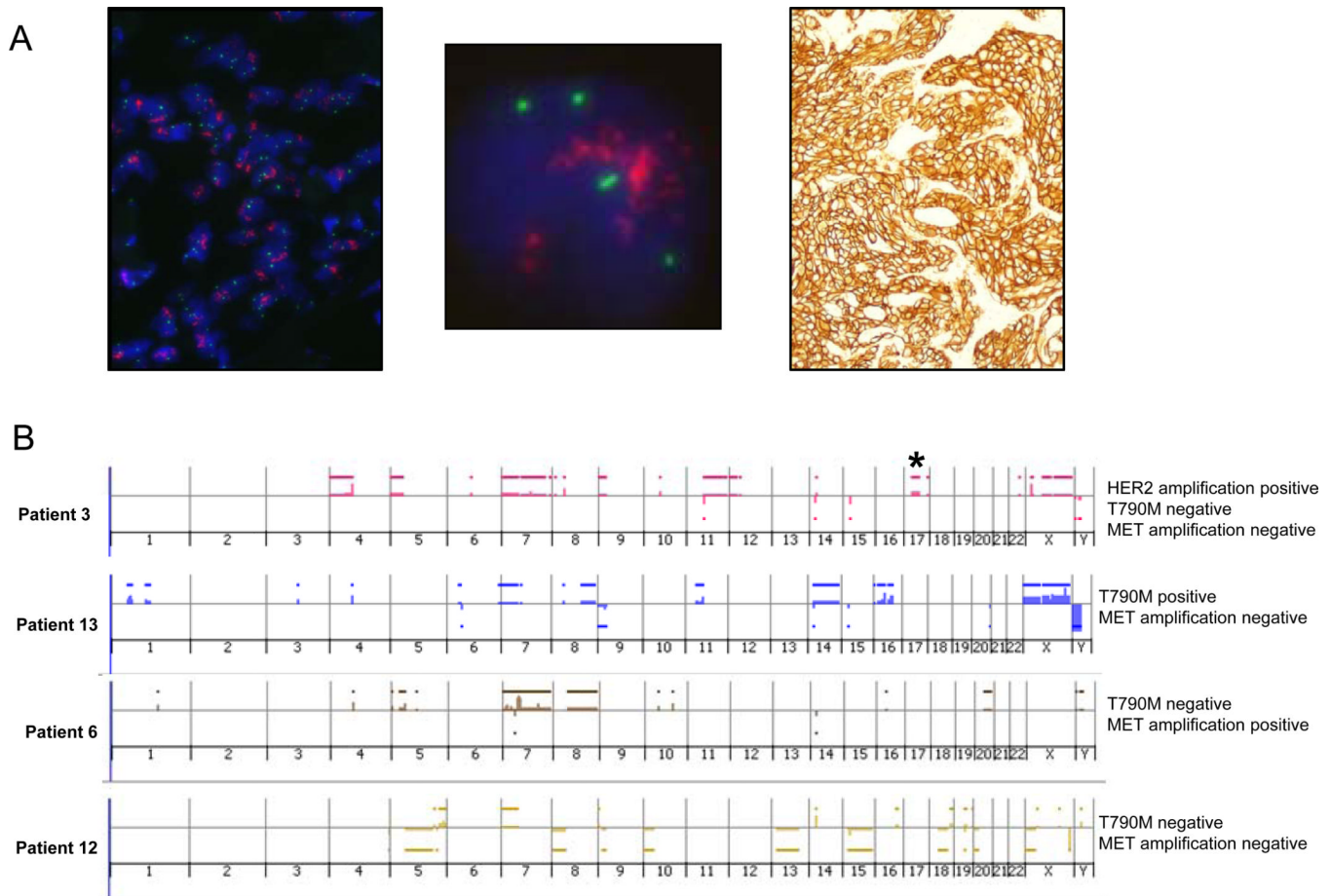
Figure 4. *HER2* amplification in EGFR T790M-negative tumors with acquired resistance
(A) *HER2* FISH (left (low magnification), middle (high magnification) panels) from a tumor specimen with acquired resistance to erlotinib. *HER2* (red) and *CEP17* (green). The PathVysion HER2 probe kit (Abbott) was 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 Her-2/chromosome17 signals > 2.2). Immunohistochemistry (right panel) was performed 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).[18] Asterisk denotes amplification in HER2, occurring in 1 of 12 samples. This sample was negative for both T790M mutation and MET amplification. Lower panels show representative examples without *HER2* amplification.
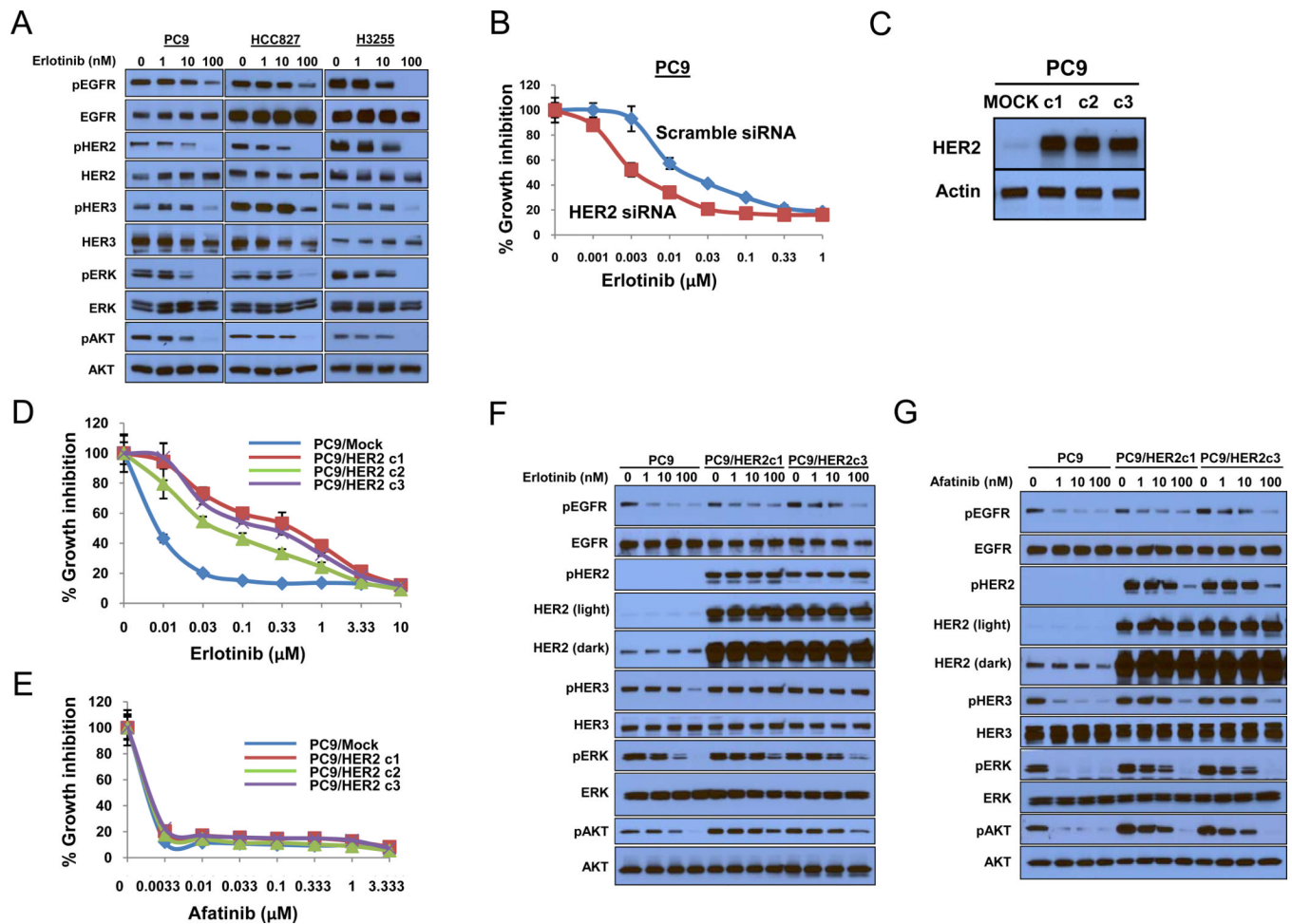
Figure 5. HER2 levels affect the sensitivity of EGFR-mutant NSCLC cells to EGFR TKIs
(A) Cells were serum starved for 12 hours prior to treatment with the indicated concentrations of erlotinib for 8 hours, after which cell lysates were subjected to immunoblot analysis with antibodies against the indicated 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 prior to treatment with afatinib for 72 hours. Points, mean of triplicates from experiments that were repeated a total of three times with similar results; bars, SD. **(C)** Cells stably transfected with either empty vector (Mock) or a *HER2* expression vector (clones 1–3) were harvested and subjected to immunoblot analysis with antibodies to the indicated proteins. **(D, E)** Cells were incubated for 24 hours prior to 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 a total of three times with similar results; bars, SD. **(F, G)** Cells were serum starved for 12 hours prior to 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.
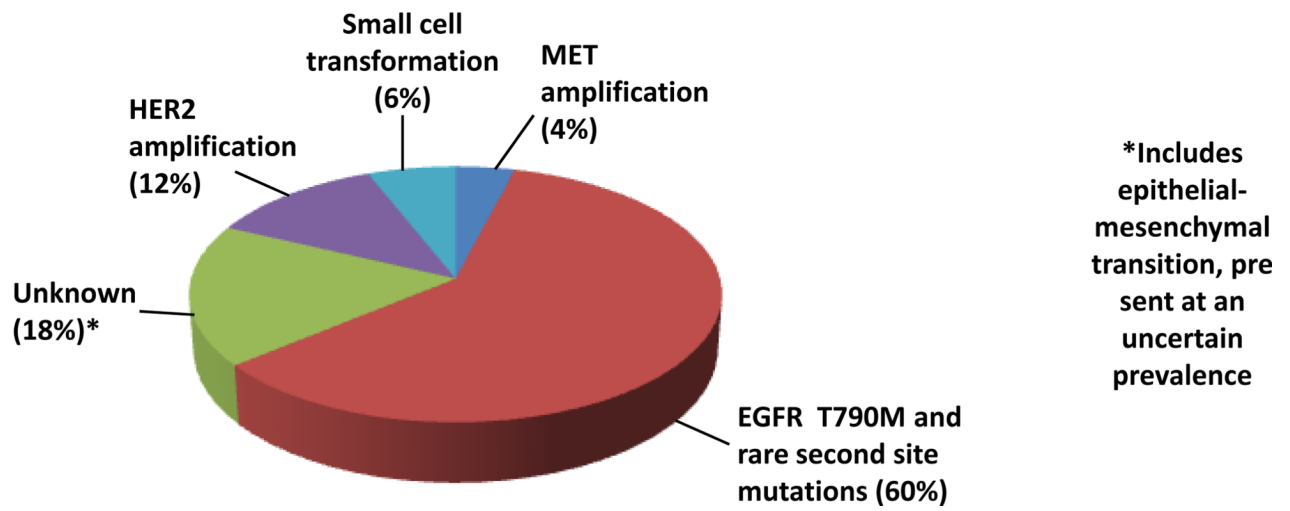
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Figure 6. Frequency of mechanisms of acquired resistance to EGFR-TKIs in EGFR mutant lung cancer

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