Cancer Therapy: Preclinical

Dual Kinase Inhibition of EGFR and HER2 Overcomes Resistance to Cetuximab in a Novel *In Vivo* Model of Acquired Cetuximab Resistance

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Abstract

Purpose: Acquired resistance to cetuximab, a chimeric epidermal growth factor receptor (EGFR)–targeting monoclonal antibody, is a widespread problem in the treatment of solid tumors. The paucity of preclinical models has limited investigations to determine the mechanism of acquired therapeutic resistance, thereby limiting the development of effective treatments. The purpose of this study was to generate cetuximab-resistant tumors *in vivo* to characterize mechanisms of acquired resistance.

Experimental Design: We generated cetuximab-resistant clones from a cetuximab-sensitive bladder cancer cell line *in vivo* by exposing cetuximab-sensitive xenografts to increasing concentrations of cetuximab, followed by validation of the resistant phenotype *in vivo* and *in vitro* using invasion assays. A candidate-based approach was used to examine the role of HER2 on mediating cetuximab resistance both *in vitro* and *in vivo*.

Results: We generated a novel model of cetuximab resistance, and, for the first time in the context of EGFR-inhibitor resistance, we identified increased phosphorylation of a C-terminal fragment of HER2 (611-CTF) in cetuximab-resistant cells. Afatinib (BIBW-2992), an irreversible kinase inhibitor targeting EGFR and HER2, successfully inhibited growth of the cetuximab-resistant cells *in vitro*. When afatinib was combined with cetuximab *in vivo*, we observed an additive growth inhibitory effect in cetuximab-resistant xenografts.

Conclusions: These data suggest that the use of dual EGFR-HER2 kinase inhibitors can enhance responses to cetuximab, perhaps in part due to downregulation of 611-CTF. This study conducted in a novel *in vivo* model provides a mechanistic rationale for ongoing phase I clinical trials using this combination treatment modality. *Clin Cancer Res; 17(18); 5935–44. ©2011 AACR.*

Introduction

The epidermal growth factor receptor (EGFR) is expressed in many solid tumor types including colorectal, lung, breast, pancreas, bladder, and head and neck cancers. EGFR signaling is involved in diverse cellular processes including growth, differentiation, and survival during tumorigenesis (1). EGFR is commonly targeted either by small-molecule tyrosine kinase inhibitors specific to EGFR such as gefitinib or erlotinib or by a chimeric human-mouse monoclonal antibody, cetuximab. EGFR is known

to be overexpressed in bladder cancers, and several immunohistochemical studies have correlated EGFR expression with poor prognosis (2). A phase II trial combining cetuximab with standard chemotherapies is currently underway in bladder cancer (3). In other epithelial cancers such as head and neck cancer, cetuximab is known to provide a clinical benefit when used in conjunction with radiation alone or in combination with chemotherapy (4), but the response rate to cetuximab as a monotherapy is modest (as low as 10%–13%; ref. 5). Compensatory mutations such as activating *K-ras* mutations, gatekeeper mutations (*T790M*) in the tyrosine kinase domain of EGFR, and EGFRvIII (a constitutively active, truncated form of EGFR lacking an extracellular domain) are not ubiquitous across cancer types but are known to contribute to resistance to EGFR-targeted therapies in certain cancer types including lung cancer, colon cancer, and glioma (6–8). To date, no consistent mechanism of resistance to cetuximab has been identified in cancers that lack these mutations including epithelial cancers such as bladder cancer and head and neck cancer (9–11). This is likely a result of both the scarcity of tumor specimens from cancer patients following treatment

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Translational Relevance

Acquired resistance to the Food and Drug Administration–approved epidermal growth factor receptor (EGFR)–targeting antibody cetuximab is a major problem in the treatment of several solid tumor types that lack mutations known to confer cetuximab resistance. Phase I clinical trials are currently underway to test whether the addition of dual kinase inhibitors targeting EGFR and HER2 to cetuximab treatment is a plausible way to increase its therapeutic efficacy. This study shows the *in vivo* efficacy of this treatment regimen in a novel preclinical model of cetuximab resistance, in addition to providing a novel biochemical mechanism in support of such trials.

with cetuximab and the paucity of preclinical models available to study mechanisms of cetuximab resistance.

One possible mechanism of cetuximab resistance, including alternative translation initiation of HER2, may involve redundant signaling through other ErbB family members (HER reprogramming). Coexpression of multiple ErbB family members is more predictive of shortened survival than expression of EGFR alone (12), and coactivation of EGFR with HER2 has been implicated in resistance to trastuzumab, a HER2-targeting agent, in breast cancer models (13). EGFR is also shown to be upregulated after long-term exposure to trastuzumab (14), further reinforcing the critical nature of these redundant pathways to cellular growth in malignancies. Trastuzumab has been shown to resensitize lung cancer cells to cetuximab *in vitro* (15), likely because HER2 signaling occurs through many of the same downstream effectors as EGFR including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (16).

Although cetuximab produces strong antitumor effects on human cancer cells *in vivo* (17, 18), it has suboptimal antiproliferative effects *in vitro* (19, 20) and is best modeled *in vitro* using invasion assays (21). In the present study, we generated an *in vivo* model of cetuximab resistance. This *in vivo* generated model of cetuximab resistance provides a means to biochemically examine relevant mechanisms of resistance. Furthermore, this model may be used to test the targeting of such resistance mechanisms *in vivo* to overcome resistance to cetuximab. Here, for the first time in the context of resistance to an EGFR-targeting agent, we describe increased phosphorylation of 611-CTF, a truncated fragment of HER2, in our cetuximab-resistant model. We also show *in vivo* that combined inhibition of EGFR and HER2 with a dual kinase-targeting agent can overcome resistance to cetuximab.

Materials and Methods

Cells and reagents

SCC1 was derived from a primary HNSCC tumor, and both SCC1 and the cetuximab-resistant clone SCC1c8 were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10% FBS and 0.4 µg/mL hydrocortisone (15). OSC-19 cells were maintained in Minimum Essential Medium with 10% FBS and 1% nonessential amino acids. CAL33, T24, and A431 cells were maintained in DMEM + 10% FBS. All cell lines were validated by genotyping within 6 months of their use with the AmpFlSTR Identifiler System (Applied Biosystems). Cetuximab-resistant clones were maintained in media with 100 nmol/L cetuximab. Cetuximab (Erbitux; ImClone Systems and Bristol-Myers Squibb) was purchased from the School of Pharmacy, University of Pittsburgh. Afatinib was obtained from Boehringer Ingelheim as a powder and resuspended in dimethyl sulfoxide for *in vitro* studies or 0.5% methylcellulose with 0.4% Tween 80 in saline for animal studies. Trastuzumab (Herceptin; Genentech) was purchased from the School of Pharmacy, University of Pittsburgh, and diluted as recommended in the package insert. Erlotinib was purchased from ChemieTek.

In vivo model generation

Subcutaneous xenografts were generated from 6 different epithelial cancer cell lines (T24, CAL33, A431, OSC-19, SCC1, and SCC1c8; $n = 6$ for all cell lines except T24, where $n = 12$) in athymic nude mice, using 1×10^6 cells with Matrigel (BD Biosciences). After tumor formation (7–10 days), mice received 0.8 mg of cetuximab by intraperitoneal (i.p.) injection twice weekly. Tumors were measured twice weekly. If tumors progressed after 14 days of treatment, dosing was increased to 1.0 mg of cetuximab twice weekly and then 0.8 mg of cetuximab 3 times per week after 28 days. If no tumors were present, the animal was sacrificed after 90 days of treatment. If tumors were present, the animal was sacrificed at 90 days or when the tumor diameter exceeded 20 mm. Tumors were removed, digested, and suspended as single cells, which were propagated in culture and reinoculated as 2 subcutaneous xenografts. These tumors were treated with 0.8 mg of cetuximab 3 times per week immediately following tumor formation.

Animal studies

For the differential sensitivity study, 1×10^6 parental and resistant cells were blindly injected on opposite flanks of the same mouse ($n = 7$) with Matrigel. Treatment began following tumor formation. Animals were treated with 2.0 mg of cetuximab 3 times weekly by i.p. injection. For the combination study, 2×10^6 parental and resistant cells were injected on opposite flanks of the same mouse ($n = 40$) with Matrigel, and animals were stratified by tumor volume (22) into 4 groups and then randomly distributed from each group into 4 treatment groups with 10 animals per group. Animals were treated with cetuximab, afatinib, or both. The treatments and measurements were conducted by an individual blinded to the treatment. One milligram of cetuximab or vehicle control was given by i.p. injection 3 times weekly, and 0.4 mg afatinib or vehicle control was given daily by oral gavage. *P* values were generated using a Mann–Whitney test for nonparametric data.

Invasion assay

Five thousand cells were plated in the inner well of a Matrigel invasion chamber (BD Biosciences) in serum-free media. Wells were placed into media containing 10% FBS, and drugs were added to both chambers where indicated. After 24 hours, cells invading through the Matrigel-coated membrane were stained and counted. P values were generated using a homoscedastic 2-tailed Student's t test.

Immunoprecipitations and Western blotting

Immunoblots were carried out on cell lysates collected 48 hours after plating in drug-free media. Lysates were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes prior to antibody staining with the following antibodies: EGFR (BD Transduction Laboratories); HER2 and 611-CTF (clone F11, sc-7301; Santa Cruz); pHER2 and p611-CTF at Y1248 [(2247s); Cell Signaling]; p-serine (BD Transduction Laboratories); and cortactin (Upstate Biotechnology). Densitometry was carried out using ImageJ software, and P values were generated by a Student's t test.

Apoptosis testing

Tumors were initially snap frozen then fixed in 4% paraformaldehyde overnight, followed by 30% sucrose overnight before embedding and cryosectioning. Tissue sections were stained using the TumorTACS Apoptosis Detection Kit (Trevigen) on the basis of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining and according to the manufacturer's protocol.

Short hairpin RNA experiments

Lentiviral particles were provided by Dr. R.W. Sobol and the University of Pittsburgh Cancer Institute (UPCI) Lentiviral Facility. Virus stocks were generated by cotransfection of the short hairpin RNA (shRNA) expression plasmid (pLK0.1; Mission shRNA library from Sigma) into 293-FT cells together with the packaging plasmids pMD2.g (VSVG), pRSV-REV, and pMDLg/pRRE. Forty-eight hours posttransfection viral particles were collected in the culture supernatant, filtered (0.45 μmol/L), and stored at −80°C or used immediately to transduce the target cells.

Results

In vivo generation of a cetuximab-resistant preclinical model

To study mechanisms of cetuximab resistance, we created a preclinical model on the basis of the previously published in vivo generated model of trastuzumab resistance (13). Subcutaneous tumor xenografts were established using 5 cetuximab-sensitive epithelial cancer cell lines (T24, CAL33, A431, OSC-19, and SCC1) as well as a previously described cetuximab-resistant epithelial cancer cell line, SCC1c8 (15). Xenograft-bearing athymic nude mice were treated with increasing concentrations of cetuximab over the course of 3 months. Animals were initially treated with moderate doses of cetuximab that are equivalent to 4 times that of a human dose (0.8 mg 2 times/wk). This was increased to doses equivalent to 6 times the standard human dose of cetuximab (0.8 mg 3 times/wk) over the course of 3 months. A majority of the epithelial carcinoma–derived xenografts regressed with cetuximab treatment, including the head and neck cancer cell line SCC1 and its in vitro derived cetuximab-resistant clone SCC1c8 (Fig. 1A).

Although most xenografts treated with cetuximab were cetuximab-sensitive, 4 cetuximab-resistant tumors (T24PR1–4) emerged out of the 12 original xenografts from T24 bladder carcinoma cells (Fig. 1A). Cetuximab-resistant tumors T24PR1–4 were surgically removed from sacrificed animals and digested into single-cell suspensions that were used to generate cell lines of the same name in vitro and additional xenografts in vivo. Xenografts from the cetuximab-resistant cells persisted despite treatment with doses of cetuximab equivalent to 12 times the human dose of cetuximab (0.8 mg 3 times/wk) immediately upon tumor formation (Fig. 1B). The persistent growth of tumors derived from in vivo generated cetuximab-resistant cells as compared with in vitro generated cetuximab-resistant cells in high doses of cetuximab shows the validity of in vivo generation for models of drug resistance, especially for therapeutic agents such as monoclonal antibodies that are known to have antitumor effects that cannot be reproduced under cell culture conditions.

Preclinical model shows acquired resistance to cetuximab

To distinguish acquired resistance to cetuximab from intrinsic resistance, we compared cetuximab sensitivity between the cetuximab-sensitive parental cells and the cetuximab-resistant clones. To test this in vivo, athymic nude mice were inoculated with sensitive cells on one flank and resistant cells on another flank. Following tumor formation, animals were randomized on the basis of tumor volumes and treated with high concentrations of cetuximab (2.0 mg 3 times/wk). Cetuximab-sensitive tumors showed a 64.8% reduction in tumor volume on day 10 of cetuximab treatment compared with a 3.9-fold increase in cetuximab-resistant tumor volumes on day 10 of cetuximab treatment (Fig. 2A; $P = 0.002$). Frozen tumors were fixed, cryosectioned, and TUNEL-stained to detect apoptotic cells. A total of 61.7% of cells from cetuximab-sensitive tumors (T24) were apoptotic compared with only 26.3% of the cells from tumors derived from cetuximab-resistant cells (T24PR3, Fig. 2A; $P = 0.03$). These results show that by gradually increasing the dose of cetuximab in vivo over the course of 28 days, cetuximab-resistant tumors can be generated.

To show the differential cetuximab sensitivity of this model in vitro, we conducted invasion assays, as cetuximab does not inhibit proliferation in vitro (20). Cetuximab has been previously reported by us and others to successfully decrease cell invasion through a Matrigel-coated Transwell
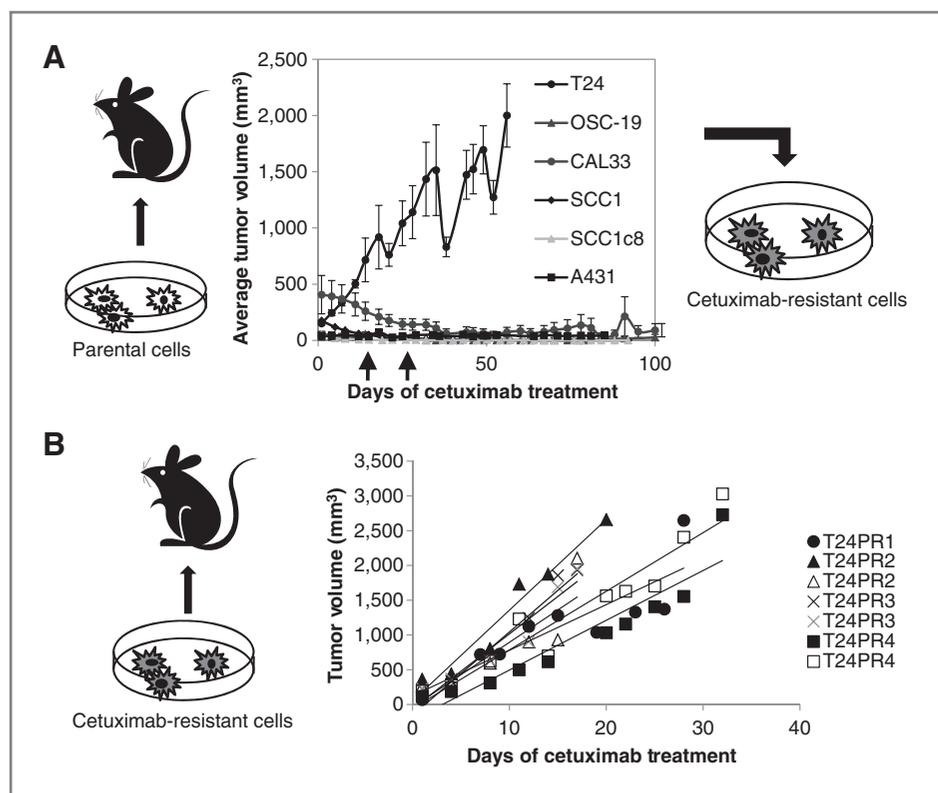
Figure 1. Generation of a cetuximab resistance model *in vivo*. A, T24, CAL33, A431, OSC-19, SCC1, and SCC1c8 cells were used to generate xenografts in athymic nude mice ($n = 6$ for CAL33, A431, OSC-19, SCC1, and SCC1c8; $n = 12$ for T24) that were exposed to increasing concentrations of cetuximab by i.p. injection (0.8 mg 2×/wk increased to 1.0 mg 2×/wk and then increased to 0.8 mg 3×/wk; doses increased in nonresponsive xenografts only as indicated by arrows). Resistant tumor cells were then harvested and propagated in culture and (B) reinoculated to form xenografts that were treated with 0.8 mg 3×/wk immediately following tumor formation.

migration chamber (23, 24). In this model, cetuximab decreased the invasion of parental T24 cells by 55.5% after 24 hours. In contrast, cetuximab only inhibited the invasion of T24PR3 and T24PR4 cells by 1.7% ($P = 0.0009$) and 8.7% ($P = 0.0001$), respectively (Fig. 2B).

## Cetuximab-resistant cells express hyperphosphorylated 611-CTF

We used a candidate-based approach to explore differences in the cetuximab-sensitive and cetuximab-resistant cells, focusing primarily on the expression and phosphorylation of ErbB family members. Consistent with other *in vitro* studies of cetuximab resistance (25), EGFR was downregulated in cetuximab-resistant T24PR3 and T24PR4 cells compared with the isogenic parental T24 cells and the other cetuximab-sensitive cell lines used in this study (Fig. 3A). HER3 was expressed at low levels in T24, T24PR3, and T24PR4 clones, and we observed no significant difference in expression of total or phosphorylated levels of HER3 across these cell lines (data not shown). Furthermore, although there was no significant change in the expression or phosphorylation status of full-length HER2 among cetuximab-sensitive and cetuximab-resistant cells, we observed a marked increase in phosphorylation of 611-CTF, a C-terminal fragment of HER2 containing the transmembrane domain, in only the cetuximab-resistant cells (Fig. 3A). Despite the abundance of total 611-CTF protein in T24, T24PR3, T24PR4, and other cells, 611-CTF seems to be phosphorylated at Tyr1248, the site responsible for MAPK activation, in only the cetuximab-resistant clones

T24PR3 and T24PR4. Densitometry confirms T24PR3 and T24PR4 cells to significantly express phosphorylated 611-CTF at levels 5.6-fold ($P = 0.0223$) and 5.9-fold ($P = 0.0309$) higher, respectively, than T24 cells (Fig. 3A). Although no significant changes were observed in expression of basal or phosphorylated MAPK or AKT between the cetuximab-sensitive and cetuximab-resistant clones (data not shown), we did observe increased phosphorylation of cortactin, a known downstream target of 611-CTF (Fig. 3B; $P = 0.039$; ref. 26).

Targeting 611-CTF can restore sensitivity to cetuximab *in vitro*

To determine the functional role of phosphorylated 611-CTF in mitigating resistance to cetuximab, we treated T24PR3 cells with cetuximab and HER2 shRNA or various HER2-targeting agents. First, we used lentiviral shRNA transduction to knockdown full-length HER2 and 611-CTF in 4 separate clones of T24PR3 (Fig. 4A). HER2 knockdown in clones 2 and 4 reduced full-length HER2 by 70% and 78%, respectively, compared with nontargeting scrambled shRNA–transduced control cells. Likewise, HER2 knockdown in clones 2 and 4 reduced 611-CTF expression by 46% and 56%, respectively, compared with scrambled shRNA–transduced cells. This HER2 knockdown of full-length HER2 and 611-CTF could restore the effect of cetuximab on T24PR3 cells in culture. Cetuximab decreased invasion of the HER2 shRNA–transduced cells by 54.9% ($P = 0.047$) and 49.5% ($P = 0.034$), respectively, after 24 hours.

Figure 2. Validation of cetuximab resistance model *in vitro* and *in vivo*. A, xenografts were generated by subcutaneous inoculation of 1 million tumor cells in athymic nude mice ($n = 7$) from cetuximab-sensitive T24 or cetuximab-resistant T24PR3 cells and treated with cetuximab (2.0 mg $3\times$/wk by i.p. injection) immediately following tumor formation, generally at 7 to 10 days (**, $P < 0.005$). TUNEL staining was carried out on frozen, fixed tumors to detect apoptotic cells. Data shown are the average of cells counted in quadruplicate $20\times$ fields of view for 2 tumors per cell type (*, $P < 0.05$). B, invasion studies were carried out with media containing either 1 µmol/L cetuximab or drug-free media. Invasion chambers were placed in the same media containing 10% FBS. After 24 hours, invading cells were stained and counted (**, $P < 0.005$). Data are the result of 2 independent experiments run in duplicate.
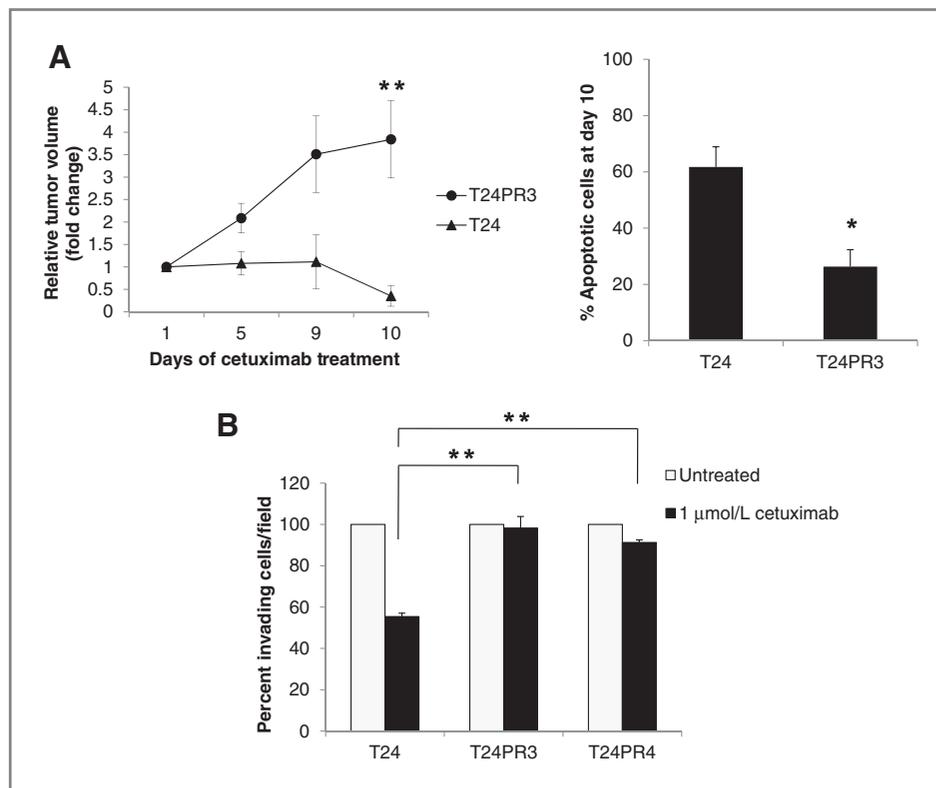
To determine whether the effects of HER2 knockdown were due to knockdown of the full-length HER2 or the 611-CTF fragment, we used HER2-targeting agents to selectively and functionally inhibit HER2 activity. Trastuzumab is a monoclonal antibody targeting exclusively full-length HER2 and should not interact directly with 611-CTF, which lacks the extracellular region containing the trastuzumab epitope (27). Although trastuzumab alone only decreased invasion of T24PR3 cells by 14.5%, the combination of cetuximab plus trastuzumab decreased invasion by 43.8% (Fig. 4B; $P = 0.01$). There is currently no kinase inhibitor available for use in the clinic that targets HER2 selectively. Afatinib is an irreversible kinase inhibitor targeting both EGFR and HER2. Afatinib is currently in phase II trials for prostate cancer, glioma, and head and neck cancer as well as phase III clinical trials for breast cancer and non–small-cell lung carcinoma (28). We found that afatinib alone could inhibit the invasion of T24PR3 cells by 38.1% (Fig. 4C; $P = 0.03$) and the combination of cetuximab plus afatinib inhibited the invasion of T24PR3 cells by 62.1% (Fig. 4C; $P = 0.031$).

Although we did not directly examine interactions between cetuximab and selective EGFR kinase inhibitors in an invasion assay, we conducted drug response assays with an EGFR kinase inhibitor using cell viability as a readout in both cetuximab-resistant and cetuximab-sensitive cells. The cetuximab-resistant and cetuximab-sensitive cells showed similar IC$_{50}$ values to the EGFR kinase inhibitor erlotinib, 6.37 µmol/L and 9.99 µnmol/L, respectively

($P = $ nonsignificant). In contrast, the IC$_{50}$ of cetuximab-resistant cells treated with afatinib was 8.27 nmol/L. These data suggest that cotargeting EGFR with a dual-specificity tyrosine kinase inhibitor that can also inhibit HER2 and 611-CTF may enhance the effects of EGFR targeting alone *in vitro* in a cetuximab-resistant cell model.

Dual kinase inhibition of EGFR and HER2 enhances antitumor effects of cetuximab *in vivo*

To test the effects of EGFR-HER2 dual kinase inhibition on mediating cetuximab sensitivity *in vivo*, we generated xenografts in athymic nude mice by inoculating cetuximab-sensitive cells on one flank and cetuximab-resistant cells on the other flank of the same mouse. Following tumor formation, animals were randomized on the basis of tumor volumes and treated with vehicle control, cetuximab alone, afatinib alone, or cetuximab plus afatinib. After 21 days, the treatment regimen of cetuximab plus afatinib yielded a 76.5% reduction in cetuximab-resistant tumor volumes ($P = 0.0191$) compared with vehicle control–treated tumors (Fig. 5A). A similar reduction in tumor volumes was seen in cetuximab-sensitive tumors treated with cetuximab and afatinib (89.7%, Fig. 5B; $P = 0.0191$), although no additional benefit was observed from adding afatinib to cetuximab therapy in cetuximab-sensitive xenografts because of the already potent antitumor effects of cetuximab on these tumors. The difference in tumor volumes between the cetuximab-sensitive and cetuximab-resistant
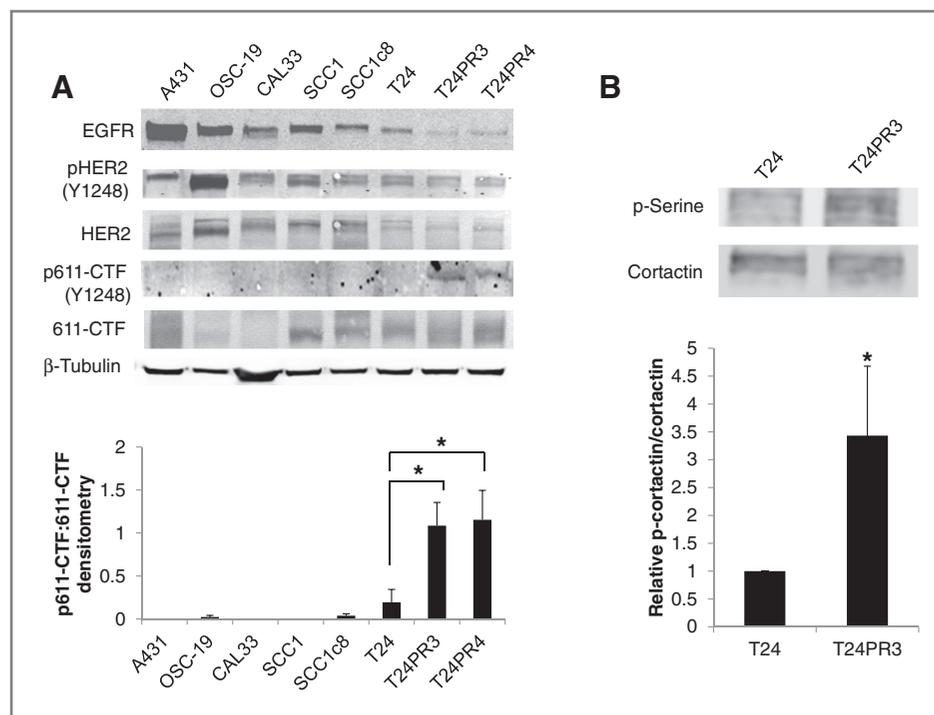
Figure 3. 611-CTF is hyperphosphorylated in cetuximab-resistant cells. A, cell lysates were collected under basal conditions when cells reached 70% confluence. Whole lysates were analyzed by Western blotting and probed for EGFR, HER2, and pHER2 (185 kDa) and 611-CTF and p611-CTF (110-kDa HER2 fragment). Densitometry is the result of 3 individual experiments where intensity of the p611-CTF bands was compared with the intensity of 611-CTF bands on the same gel (*, $P < 0.05$). A full image of these gels is available (Supplementary Fig. S1). B, cell lysates were collected under basal conditions when cells reached 70% confluence. Whole lysates were analyzed by Western blotting and probed for p-serine and cortactin. Densitometry is the result of 6 individual experiments where intensity of the p-serine bands for each cell type was compared with their respective cortactin bands from the same gel. Phosphorylation ratios for each cell line were compared with the T24 cell line for reference (*, $P < 0.05$).

xenografts treated with cetuximab was again significant ($P = 0.0013$), as shown earlier with a higher dose of cetuximab (Fig. 2A). Interestingly, 611-CTF expression in the cetuximab-resistant tumors was significantly increased in tumors treated with cetuximab alone but decreased in those treated with the combination of afatinib and cetuximab (Fig. 5C; $P = 0.015$ and $P = 0.0047$, respectively). 611-CTF expression is slightly increased in the afatinib-treated tumors, although this difference was not statistically significant (Fig. 5C; $P = 0.11$). Furthermore, the dramatic reduction in cetuximab-resistant tumor volumes that was seen with the combination of cetuximab plus afatinib far surpasses the effect observed when either agent was used as a monotherapy, which suggests that dual kinase inhibition of EGFR and HER2 may be an effective way to enhance the efficacy of cetuximab *in vivo* in the context of acquired resistance.

Discussion

Acquired resistance to cetuximab is an important clinical problem in cancer patients treated with this Food and Drug Administration–approved EGFR monoclonal antibody. Elucidation of the mechanisms of acquired resistance has been limited by the paucity of preclinical models. In the present study, we examined the *in vivo* response to cetuximab in a panel of xenografts derived from epithelial carcinomas in which activation of HER2 was detected in the cetuximab-resistant tumors. Further investigation showed that treatment of cetuximab-resistant tumors with a dual kinase inhibitor specific for EGFR and HER2 overcame cetuximab resistance. Previous attempts to generate an *in vivo* model of cetuximab resistance could not culture cells from their cetuximab-resistant xenografts (19). Another group has successfully generated *in vitro* models of cetuximab resistance, although *in vivo* validation with statistical support is lacking (15, 29, 30). In contrast, the model presented in the current study was generated *in vivo* and shown to be statistically significant *in vivo* across several doses of cetuximab including 1.0 mg 3 times/wk and 2.0 mg 3 times/wk. These more robust dosing schedules were chosen because they are higher than the therapeutic human dose, they are used widely by others in the literature (7, 31), and doses greater than 0.25 mg 3 times/wk have been previously identified as the optimal therapeutic doses of cetuximab in pharmacokinetic studies using mice (32). Furthermore, one group initially reported *in vitro* generated models of trastuzumab resistance and subsequently reported that these models were not reproducible

Figure 4. A, T24PR3 cells were transduced with HER2 shRNA–containing lentiviral particles, and a representative image of the Western blots for full-length HER2 and 611-CTF from 4 clones and scramble control lysates are included here. Invasion studies in this figure were conducted as described earlier (*, $P < 0.05$), and all data are the result of 2 independent experiments run in duplicate. B, invasion studies were conducted using T24PR3 cells with media containing vehicle, cetuximab, trastuzumab to inhibit full-length HER2, or both cetuximab and trastuzumab. C, invasion studies were conducted using T24PR3 cells with media containing vehicle, cetuximab, the EGFR-HER2 kinase inhibitor afatinib, or both cetuximab and afatinib.
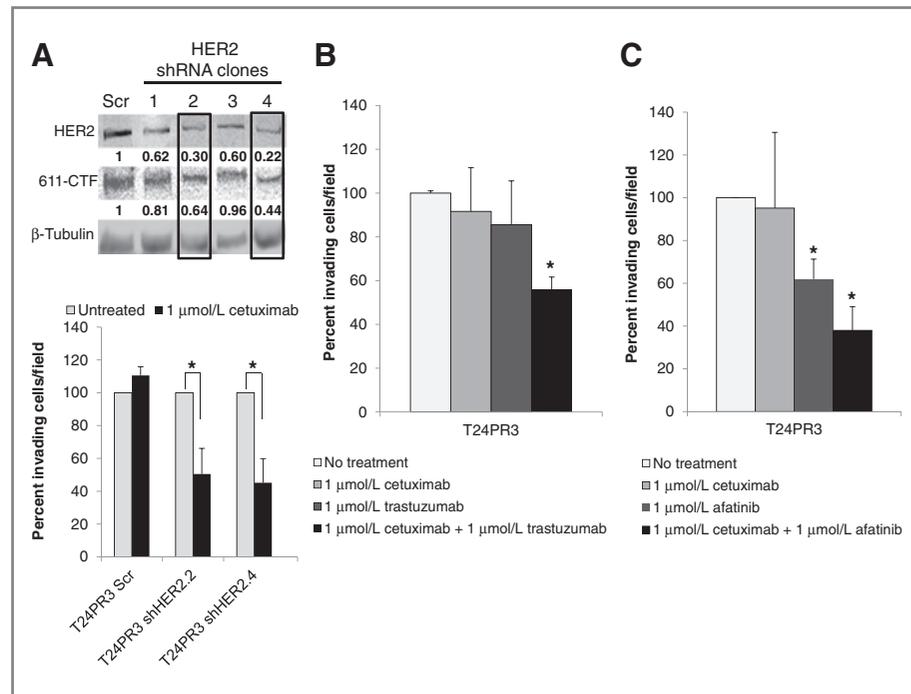
in vivo, suggesting that *in vitro* generated models of antibody resistance may not extend to *in vivo* settings and underscoring the importance of generating models of resistance to biological therapeutics *in vivo* (33).

It is worth noting that the T24 model has been previously reported to contain an *H-ras*–activating mutation (34). Given the extensive evidence that *K-ras* mutations confer resistance to cetuximab in colon cancer (35), the contribution of the *H-ras* mutation to the cetuximab resistance mechanisms described in the present study remain unknown.

HER kinase receptor switching has been described as a major determinant of acquired resistance to inhibition of these receptors (36). For this reason, we decided to examine the expression and activation of other ErbB family members. Although we observed no marked differences in ErbB family expression or activation across the 5 cell lines tested *in vivo*, the HER2 fragment 611-CTF was most robustly expressed in the T24 cell line and the SCC1 cell line, which was used to generate the previously published *in vitro* model of cetuximab resistance. Our results implicate 611-CTF in cetuximab resistance, namely, that therapeutic targeting of both HER2 and 611-CTF is highly efficacious *in vitro* and *in vivo* (Figs. 4C and 5A and B) and that expression of 611-CTF is lost in tumors treated with cetuximab plus afatinib (Fig. 5C).

The exact mechanism of the increased antitumor activity seen with the addition of afatinib to cetuximab and to what extent inhibiting 611-CTF plays a role in this mechanism remains incompletely understood. 611-CTF has been described as a 110-kDa alternative translation product of HER2 containing the C-terminal, intracellular and transmembrane domains along with a truncated extracellular domain (37). It has been shown to promote tumor growth

and metastasis in breast cancer cells *in vivo* (38) and has also been implicated in cell motility and invasiveness (26), further enforcing its metastatic function. Interestingly, 611-CTF is thought to be hyperactive because of its ability in models of forced expression to constitutively homodimerize through disulfide bonds as a result of unbalanced extracellular cysteine residues (38). The endogenous presence of nonphosphorylated 611-CTF in several cell lines including A431, SCC1, SCC1c8, and T24 cells (Fig. 3A) suggests that 611-CTF is not always a constitutively active HER2 fragment and that it may require heterodimer partners for activation under some conditions. ErbB receptor heterodimerization might also explain, in part, the antitumor activity of the trastuzumab/cetuximab combination in the cetuximab- and trastuzumab-resistant T24PR3 model. One limitation of the present study is that the precise mechanism of resistance and 611-CTF activation could not be elucidated because of the low levels of endogenous 611-CTF expression in our cell lines, and our attempts at cloning constitutively active and kinase dead forms of 611-CTF for forced expression studies have been unsuccessful to date.

Combinatorial treatment regimens are currently at the forefront of growth factor molecular targeting (36, 39). Two recent preclinical reports describe the *in vivo* benefit of combining cetuximab with kinase inhibitors specific for EGFR and/or HER2, although in both cases, the work was carried out in the context of an acquired mutation (*T790M*) that confers resistance to the EGFR tyrosine kinase inhibitor gefitinib (40, 41). Both these studies and the current one provide complementary data supporting the use of a treatment regimen that is particularly timely and pertinent with
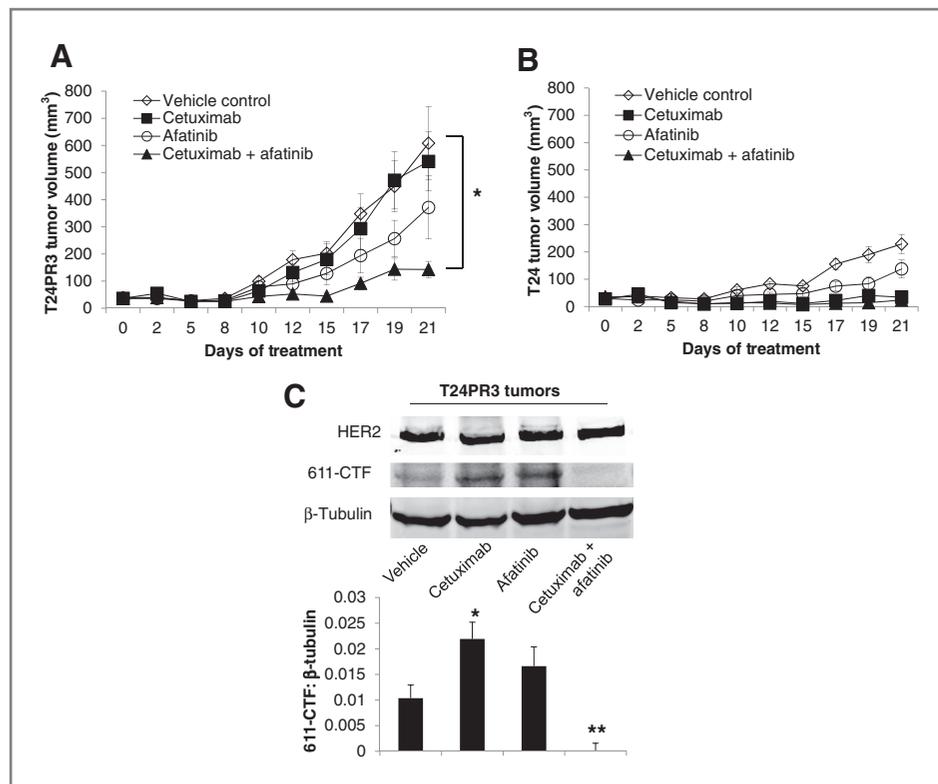
Figure 5. Dual kinase inhibition of EGFR and HER2 in vivo. Xenografts were created using cetuximab-resistant T24PR3 cells (A) or cetuximab-sensitive T24 cells (B) in athymic nude mice ($n = 40$). Mice were randomized on the basis of tumor volumes and treated with vehicle control, afatinib (0.4 mg daily by oral gavage), cetuximab (1.0 mg 3×/wk by i.p. injection), or both drugs concurrently for 21 days. Tumor volumes were measured 3 times per week for a total of 3 weeks. P values were generated using a Mann–Whitney test (*, $P < 0.05$). C, cell lysates were created from snap-frozen tumor tissue. Whole lysates were analyzed by Western blotting and probed for 611-CTF. Densitometry is the result of 4 technical replicates from 1 individual tumor of each treatment group normalized to the β-tubulin loading control for each tumor (*, $P < 0.05$; **, $P < 0.005$).

ongoing phase I clinical trials in solid tumors of afatinib plus cetuximab (ClinicalTrials.gov identifier: NCT01090011) or lapatinib plus cetuximab (ClinicalTrials.gov identifier: NCT01184482). Surprisingly, there is very little data describing molecular mechanisms in support of this treatment regimen. One recent report shows that the combination of cetuximab with lapatinib can increase antibody-dependent cell-mediated cytotoxicity (ADCC) in ^{51}Cr-release assays by up to 30% (42). The mechanism by which ADCC is increased with this treatment modality remains unknown, as the authors of the same study do not show a high frequency of lapatinib-mediated accumulation of EGFR at the cell surface as that seen for HER2 with lapatinib and trastuzumab combination treatments (43). Both studies show lapatinib-mediated accumulation of inactive HER2 at the cell surface due to loss of ubiquitination and degradation (42, 43), which may explain in part our observation that afatinib does not decrease the expression of 611-CTF in xenografts (Fig. 5C) despite decreasing tumor volume. These data are concordant with published work (43) that shows lapatinib can decrease tumor volumes in animals despite increased accumulation of HER2. Our work confirms the in vivo benefits of this combined treatment regimen, and the model presented here could be used to study the antitumor effects of ADCC in vivo in the future in addition to the other mechanisms already described here. The mouse model used in these experiments could be used to study ADCC, as others have knocked out the FCγR (found on natural killer cells, responsible for ADCC response) in nude mice and showed reduced antitumor effects of human IgG1 backbone antibodies in the FCγR$^{-/-}$ compared with FCγR$^{+/+}$ mice in the setting of treatment with trastuzumab and rituximab, which share the same IgG1 human backbone as cetuximab that is responsible for binding the FCγR and initiating ADCC (44).

The greatest limitation of the present study is the lack of human data to corroborate our findings. Unfortunately, cetuximab is currently only in phase II trials in bladder cancer, so we were unable to identify any pre- and post-treatment human bladder tissues available for investigation. Likewise, although one 611-CTF–selective antibody has been described in the literature (45), it has not been validated in other studies and no other 611-CTF–selective antibodies are commercially available to date, so there is no reliable method to examine the expression of 611-CTF in human tissues with low endogenous expression of the fragment. Recent literature using this antibody shows widespread expression of 611-CTF in a cohort of 112 breast tumors (45). This antibody has not yet been tested in bladder tumors, although a recent study (46) assessed 1,005 bladder tumors by using a cytoplasmic HER2 antibody that recognizes both full-length HER2 and 611-CTF to assess 1,005 bladder tumors and found staining in 93 (9.2%) of invasive urothelial bladder cancers.

In summary, we have successfully generated and described a novel in vivo model of cetuximab resistance, identified increased phosphorylation of 611-CTF in our resistant model, and showed that the use of a dual EGFR/HER2 kinase inhibitor can overcome resistance to cetuximab. These findings show the need for development of additional preclinical models of cetuximab resistance,

provide a platform by which to examine other mechanisms of cetuximab resistance not explored herein, and suggest a novel mechanism in support of the future trials combining cetuximab and lapatinib in solid tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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