Title

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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Support

Keywords

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Abstract

Purpose: Acquired resistance to cetuximab, a chimeric 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 in a novel *in vivo* model provides a mechanistic rationale for ongoing phase I clinical trials using this combination treatment modality.

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Statement of Translational Relevance

Acquired resistance to the FDA-approved 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. The current study demonstrates 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.

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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%) (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 with cetuximab and the paucity of pre-clinical models available to study mechanisms of cetuximab resistance.

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One possible mechanism of cetuximab resistance may involve redundant signaling through other ErbB family members (HER re-programming), including alternative translation initiation of HER2. Co-expression of multiple ErbB family members is more predictive of shortened survival than expression of EGFR alone (12) and co-activation 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 re-sensitize lung cancer cells to cetuximab *in vitro* (15), likely because HER2 signaling occurs through many of the same downstream effectors as EGFR, including MAPK and PI3K (16).

While cetuximab produces strong anti-tumor effects on human cancer cells *in vivo* (17, 18)*, it has sub-optimal anti-proliferative 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. Further, 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 demonstrate *in vivo* that combined inhibition of EGFR and HER2 with a dual kinase targeting agent can overcome resistance to cetuximab.

Materials & Methods

Cells and Reagents. SCC1 was derived from a primary HNSCC tumor and both SCC1 and the cetuximab-resistant clone SCC1c8 were maintained in DMEM with 10% FBS and 0.4ug/mL hydrocortisone (15). OSC-19 cells were maintained in MEM with 10% FBS and 1% non-essential 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 using the AmpFISTR Identifiler System (Applied Biosystems). Cetuximab-resistant clones were maintained in media with 100nM cetuximab. Cetuximab (Erbitux, ImClone Systems and Bristol-Myers Squibb) was purchased from the University of Pittsburgh Pharmacy. Afatinib was obtained from Boehringer Ingelheim as a powder and resuspended in DMSO 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 University of Pittsburgh Pharmacy 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 three 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,

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digested, and suspended as single cells, which were propagated in culture and re-inoculated as two subcutaneous xenografts. These tumors were treated with 0.8 mg of cetuximab three times per week immediately following tumor formation.

Animal Studies. For the differential sensitivity study, 1 x 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 three times weekly by i.p. injection. For the combination study, 2 x 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 four groups then randomly distributed from each group into four treatment groups with ten animals per group. Animals were treated with cetuximab, afatinib, or both. The treatments and measurements were performed by an individual blinded to the treatment. 1.0 mg of cetuximab or vehicle control was given by i.p. injection three times weekly by and 0.4 mg afatinib or vehicle control was given daily by oral gavage. P-values were generated using a Mann-Whitney test for non-parametric 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 two-tailed Student's t-Test.

Immunoprecipitations and Western Blotting. Immunoblots were performed on cell lystates collected 48h 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 Labs; HER2 and 611-CTF, (clone F11, sc-7301) Santa

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Cruz; pHER2 and 611-CTF, Y1248 (2247s) Cell Signaling; pSerine, BD Transduction Labs; Cortactin, Upstate Biotechnology. Densitometry was performed using Image J 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 prior to embedding and cryosectioning. Tissue sections were stained using the TumorTACS Apoptosis Detection Kit (Trevigen) based on TUNEL staining and according to the manufacturer's protocol.

shRNA 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 co-transfection of the shRNA expression plasmid (pLK0.1; Mission shRNA library from Sigma) into 293-FT cells together with the packaging plasmids pMD2.g (VSVG), pRSV-REV, pMDLg/pRRE. Forty-eight hours post transfection viral particles were collected in the culture supernatant, filtered (0.45 µM) and stored at -80˚C or used immediately to transduce the target cells.

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Results

***In vivo* generation of a cetuximab-resistant preclinical model**. In order to study mechanisms of cetuximab resistance, we created a preclinical model based on the previously published *in vivo* generated model of trastuzumab-resistance (13). Subcutaneous tumor xenografts were established using five cetuximab-sensitive epithelial cancer cell lines (T24, CAL33, A431, OSC-19, SCC1) as well as one 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 three months. Animals were initially treated with moderate doses of cetuximab that are equivalent to four times that of a human dose (0.8mg 2 times/week). This was increased to doses equivalent to six times the standard human dose of cetuximab (0.8mg 3 times/week) over the course of three 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 (Figure 1A).

While most xenografts treated with cetuximab were cetuximab-sensitive, four cetuximab-resistant tumors (T24PR1-4) emerged out of the twelve original xenografts from T24 bladder carcinoma cells (Figure 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 six times the human dose of cetuximab (0.8 mg 3 times/week) immediately upon tumor formation (Figure 1B). The persistent growth of tumors derived from *in vivo* generated cetuximab-resistant cells as compared to *in vitro* generated cetuximab-resistant cells in high doses of cetuximab

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demonstrates the validity of *in vivo* generation for models of drug resistance, especially for therapeutic agents such as monoclonal antibodies that are known to have anti-tumor effects that cannot be reproduced under cell culture conditions.

Preclinical model demonstrates 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 based on tumor volumes and treated with high concentrations of cetuximab (2.0 mg 3 times/week). Cetuximab-sensitive tumors demonstrated a 64.8% reduction in tumor volume on day 10 of cetuximab treatment compared to a 3.9-fold increase in cetuximab-resistant tumor volumes on day 10 of cetuximab treatment, p=0.002 (Figure 2A). Frozen tumors were harvested after ten days of cetuximab treatment, fixed, cryosectioned and TUNEL-stained to detect apoptotic cells. 61.7% of cells from cetuximab-sensitive tumors (T24) were apoptotic compared to only 26.3% of the cells from tumors derived from cetuximab resistant cells (T24PR3, Figure 2A, p=0.03). These results demonstrate that by gradually increasing the dose of cetuximab *in vivo* over the course of 28 days, cetuximab resistant tumors can be generated.

To demonstrate the differential cetuximab sensitivity of this model *in vitro,* we performed invasion assays since 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-migration chamber (23, 24). In this model, cetuximab decreased the invasion of

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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 (Figure 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 to the isogenic parental T24 cells and the other

cetuximab-sensitive cell lines used in this study (Figure 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). Further, while

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 (Figure 3A). Despite the abundance of total 611-

CTF protein in T24, T24PR3 and T24PR4 and other cells, 611-CTF appears 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 (p=0.0223) and 5.9 (p=0.0309) fold

higher, respectively, than T24 cells (Figure 3A). While no significant changes were observed in

expression of basal or phosphorylated MAPK or AKT between the cetuximab sensitive and

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cetuximab resistant clones (data not shown), we did observe increased phosphorylation of cortactin, a known downstream target of 611-CTF (Figure 3B, p=0.039)(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 four separate clones of T24PR3 (Figure 4A). HER2 knockdown in clones 2 and 4 reduced full length HER2 by 70% and 78%, respectively, compared to non-targeting scrambled shRNA-transduced control cells. Likewise, HER2 knockdown in clones 2 and 4 reduced 611-CTF expression by 46% and 56%, respectively, compared to scrambled shRNA-transduced cells. This HER2 knockdown of full length HER2 and 611-CTF was able to 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) after 24 hours.

To determine if 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% (Figure 4B, p=0.01). While there are currently no kinase inhibitors available for use in the clinic that target HER2 selectively, afatinib is an irreversible kinase inhibitor

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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% (Figure 4C, p=0.03), and the combination of cetuximab plus afatinib inhibited the invasion of T24PR3 cells by 62.1% (Figure 4C, p=0.031).

While we did not directly examine interactions between cetuximab and selective EGFR kinase inhibitors in an invasion assay, we performed 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 demonstrated similar $IC_{50}s$ to the EGFR kinase inhibitor erlotinib, 6.37μM and 9.99μM, respectively (p=n.s.). In contrast, the IC_{50} of cetuximab-resistant cells treated with afatinib was 8.27nM. These data suggest that co-targeting 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 anti-tumor effects of cetuximab *in vivo*. In order 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 based on 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

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tumor volumes (p=0.0191) compared to vehicle control treated tumors (Figure 5A). A similar reduction in tumor volumes was seen in cetuximab-sensitive tumors treated with cetuximab and afatinib (89.7%, p=0.0191) (Figure 5B), although no additional benefit was observed from adding afatinib to cetuximab therapy in cetuximab-sensitive xenografts because of the already potent anti-tumor effects of cetuximab on these tumors. The difference in tumor volumes between the cetuximab-sensitive and cetuximab-resistant xenografts treated with cetuximab was again significant (p=0.0013) as shown earlier with a higher dose of cetuximab (Figure 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 (Figure 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 (Figure 5C, p=0.11). Further, 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 FDA-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 where activation of HER2 was detected in the cetuximab resistant tumors. Further investigation demonstrated 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 were unable to 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/week and 2.0 mg 3 times/week. 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 0.25mg 3 times/week have been previously identified as the optimal therapeutic doses of cetuximab in pharmacokinetic studies using mice (32). Further, one group initially reported *in vitro* generated models of trastuzumab resistance and subsequently reported that these models were not reproducible *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).

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It is worth noting that the T24 model has been previously reported to contain an Ha-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. While we observed no marked differences in ERBB family expression or activation across the five 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* (Figure 4C, 5A-B) and expression of 611-CTF is lost in tumors treated with cetuximab plus afatinib (Figure 5C).

The exact mechanism of the increased anti-tumor 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). 611-CTF has been shown to promote tumor growth and metastasis in breast cancer cells *in vivo* (38) and it 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

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(38). The endogenous presence of non-phosphorylated 611-CTF in several cell lines including A431, SCC1, SCC1c8, and T24 cells (Figure 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 anti-tumor 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 due to 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 performed 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 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 is

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seen for HER2 with lapatinib and trastuzumab combination treatments (43). Both studies demonstrate 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 (Figure 5C) despite decreasing tumor volume. This data is 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 anti-tumor 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 NK cells, responsible for ADCC response) in nude mice and showed reduced anti-tumor effects of human IgG1 backbone antibodies in the FCγR -/- compared to 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

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breast tumors (45). This antibody has not yet been tested in bladder tumors, although a recent study (46) assessed 1005 bladder tumors using a cytoplasmic HER2 antibody that should recognize both full length HER2 and 611-CTF 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 demonstrated that using a dual EGFR/HER2 kinase inhibitor can overcome resistance to cetuximab. These findings demonstrate 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.

Acknowledgements

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Figure Legends

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 intraperitoneal injection (0.8 mg 2x/week increased to 1.0 mg 2x/week then increased to 0.8 mg 3x/week; doses increased in non-responsive xenografts only as indicated by arrows). Resistant tumor cells were then harvested and propagated in culture and (**B**) re-inoculated to form xenografts that were treated with 0.8 mg 3x/week immediately following tumor formation.

Figure 2. Validation of cetuximab resistance model *in vitro* and *in vivo*. (**A**) Xenografts were generated by subcutaneous inoculation of one 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 3x/week by intraperitoneal injection) immediately following tumor formation, generally 7-10 days (**p<0.005). TUNEL staining was performed on frozen, fixed tumors to detect apoptotic cells. Data shown is the average of cells counted in quadruplicate 20X fields of view for two tumors per cell type (*p<0.05). (**B**) Invasion studies were carried out with media containing either 1μM 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 two independent experiments run in duplicate.

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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 Blot and probed for EGFR, HER2 and pHER2 (185 kDa), 611-CTF and p611-CTF (110 kDa HER2 fragment). Densitometry is the result of three individual experiments where intensity of the p611-CTF bands were compared to the intensity of 611-CTF bands on the same gel (*$p<0.05$). A full image of these gels is available (Supplemental Figure 1). (**B**) Cell lysates were collected under basal conditions when cells reached 70% confluence. Whole lysates were analyzed by Western Blot and probed for pSerine and cortactin. Densitometry is the result of six individual experiments where intensity of the pSerine bands for each cell type were compared to their respective cortactin bands from the same gel. Phosphorylation ratios for each cell line were compared to the T24 cell line for reference (*$p<0.05$).

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 performed as described earlier (*$p<0.05$) and all data are the result of two independent experiments run in duplicate. (**B**) Invasion studies were performed using T24PR3 cells with media containing either vehicle, cetuximab, trastuzumab to inhibit full length HER2, or both cetuximab and trastuzumab. (**C**) Invasion studies were performed using T24PR3 cells with media containing either vehicle, cetuximab, the EGFR-HER2 kinase inhibitor afatinib, or both cetuximab and afatinib.

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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 based on tumor volumes and treated with vehicle control, afatinib (0.4 mg/daily by oral gavage), cetuximab (1.0 mg 3x/week by intraperitoneal 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 Blot and probed for 611-CTF. Densitometry is the result of four technical replicates from one individual tumor of each treatment group normalized to the β-tubulin loading control for each tumor (*$p<0.05$, **$p<0.005$).

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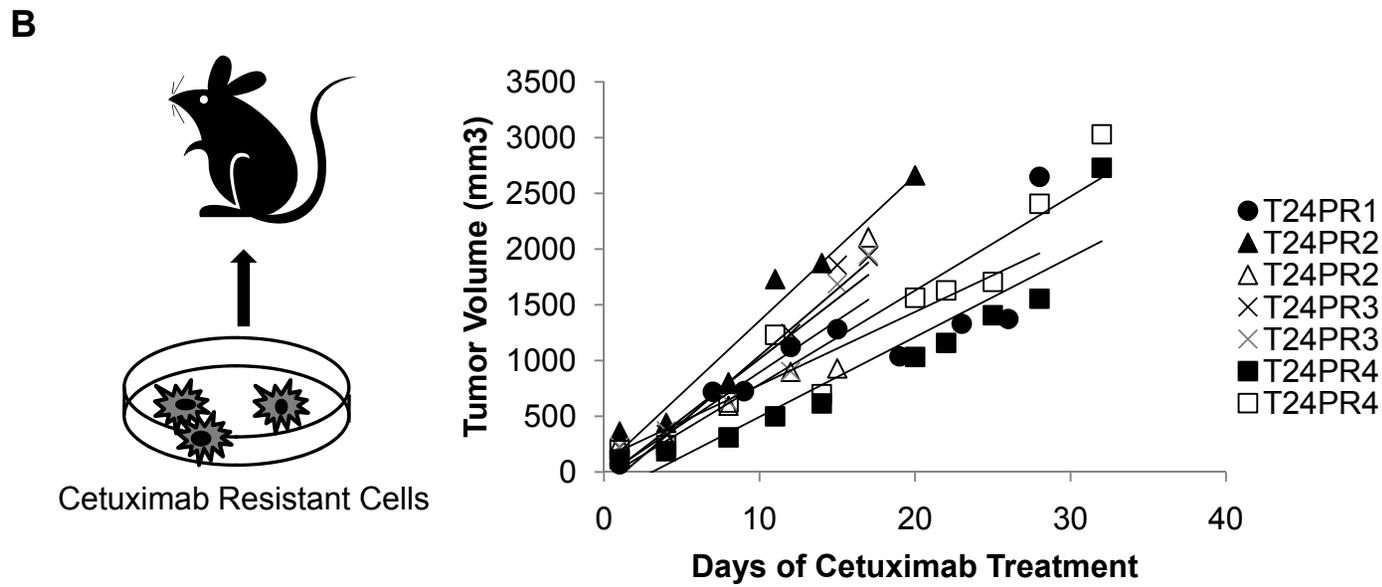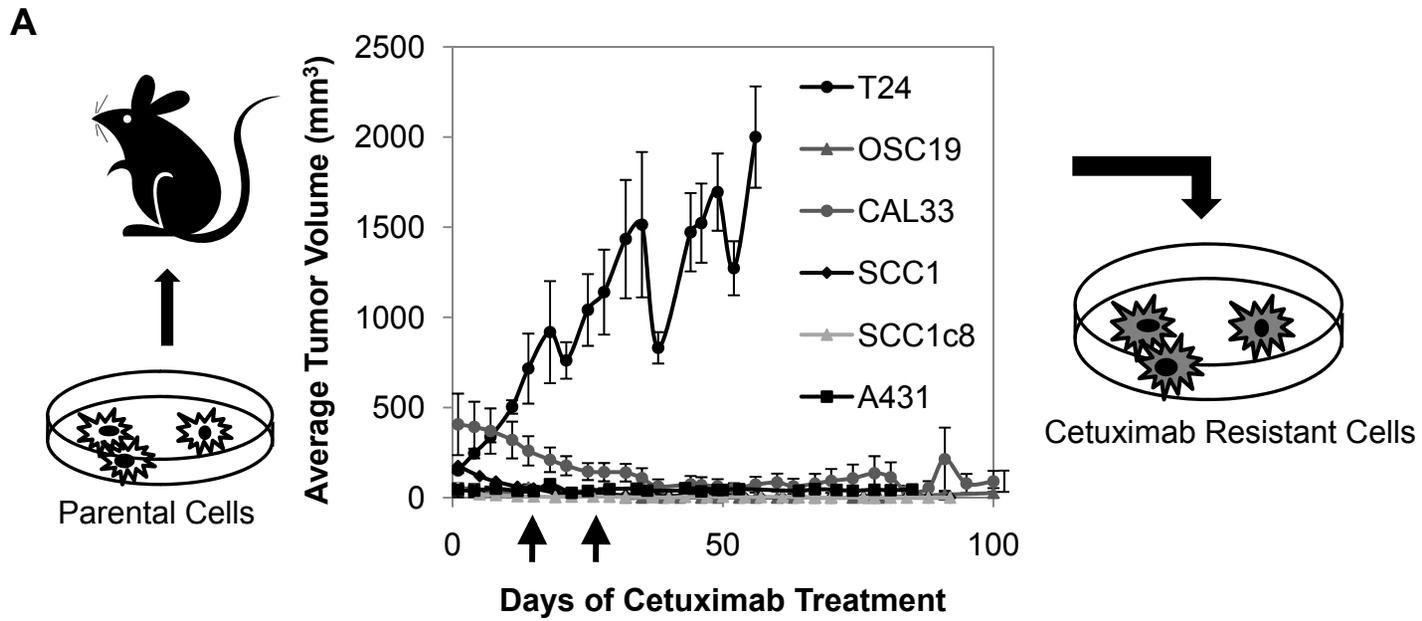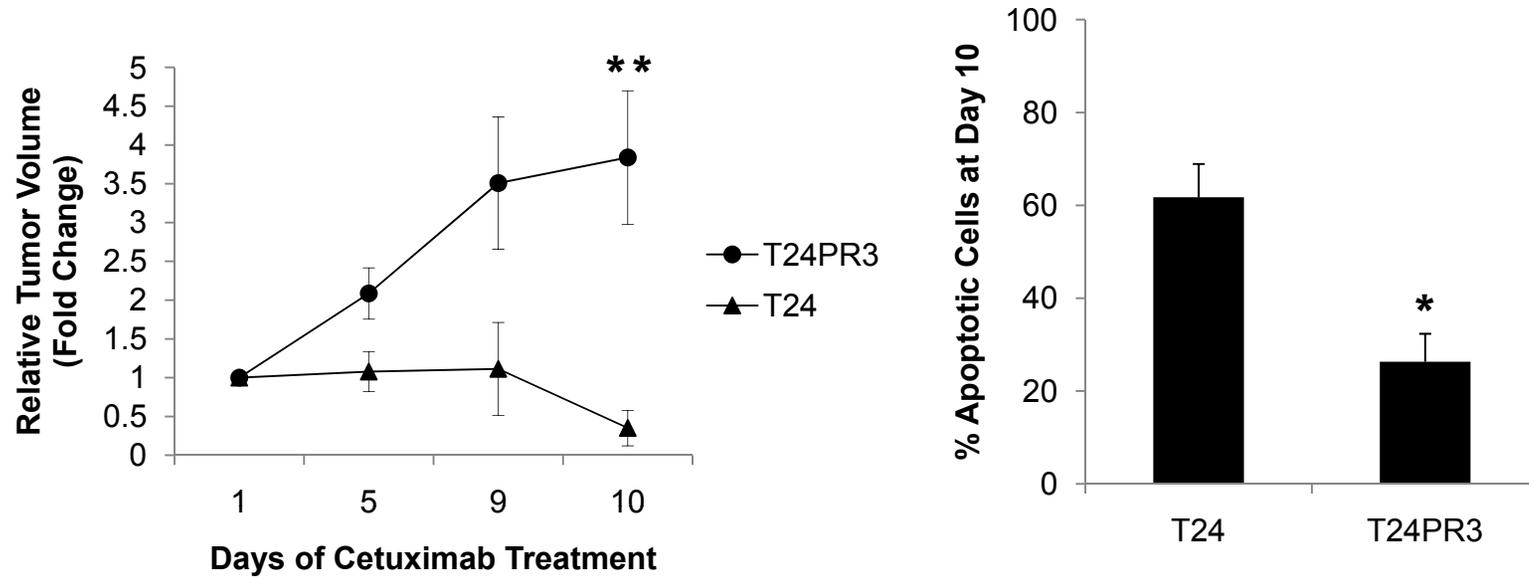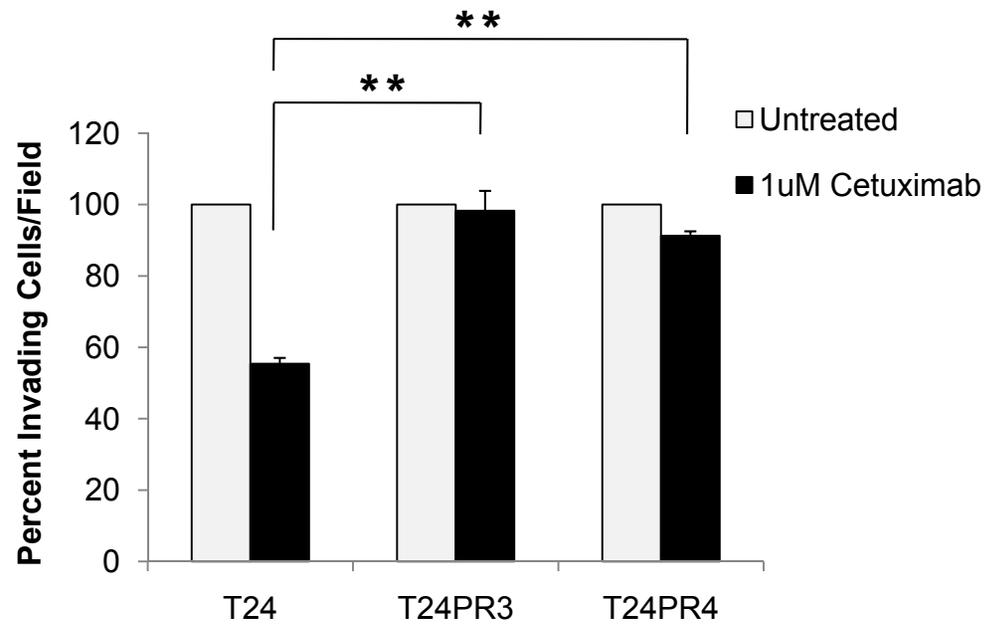
FIGURE 1

FIGURE 2

A

B

FIGURE 3

A

B

FIGURE 4
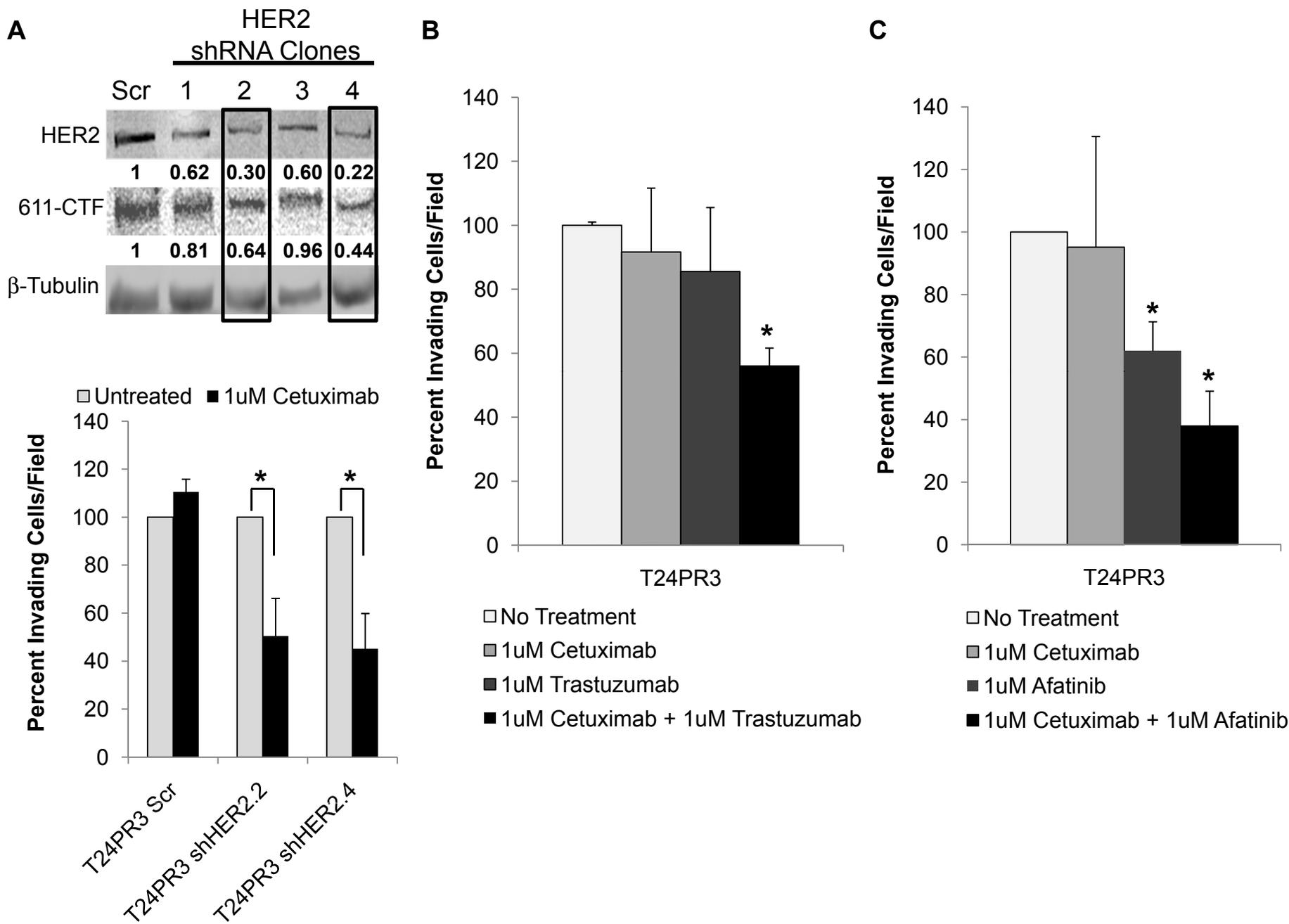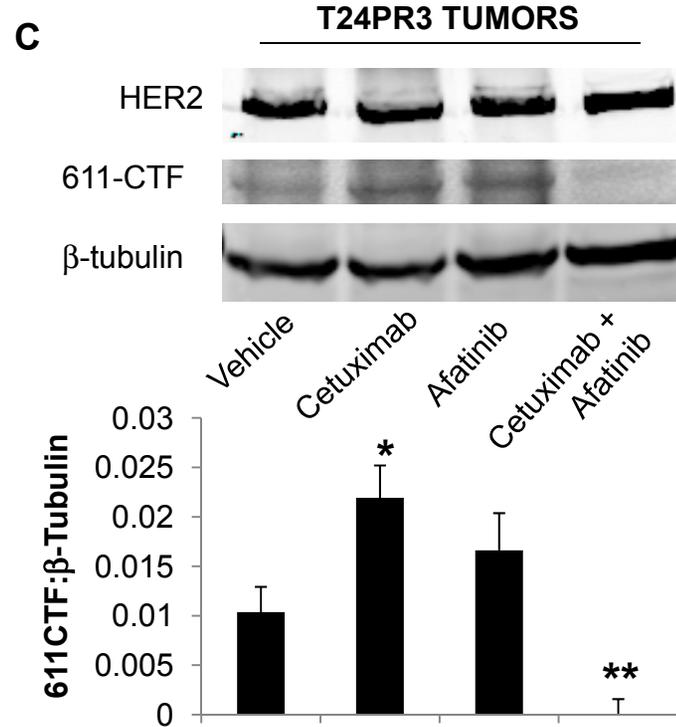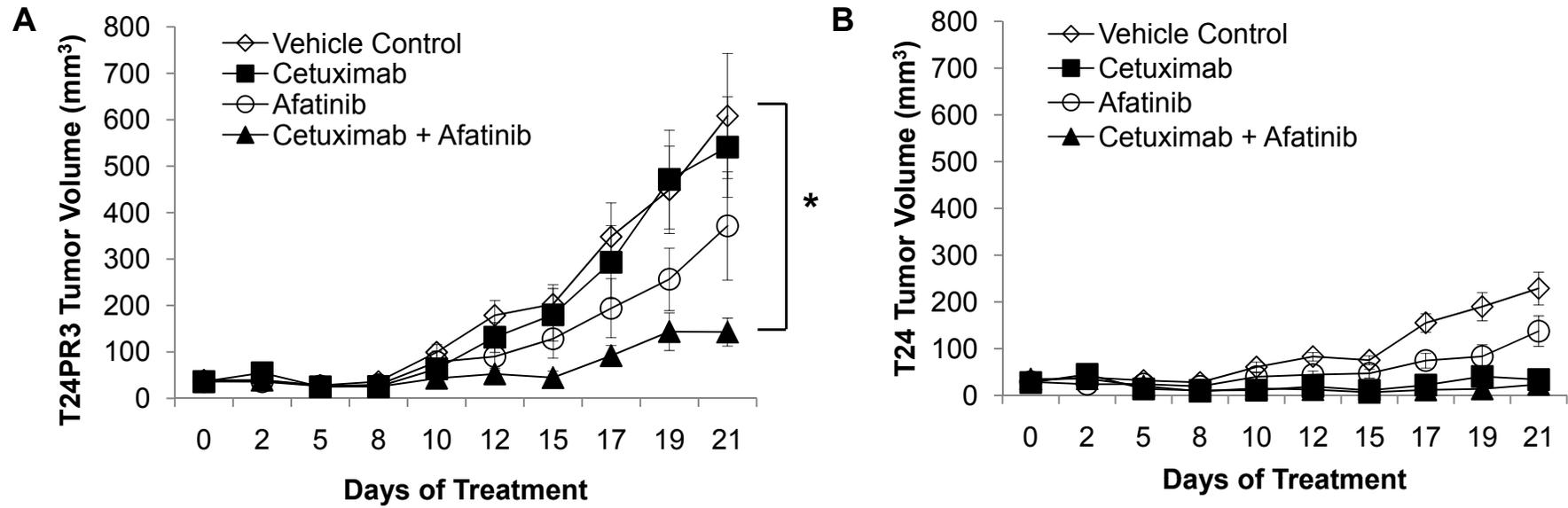
FIGURE 5

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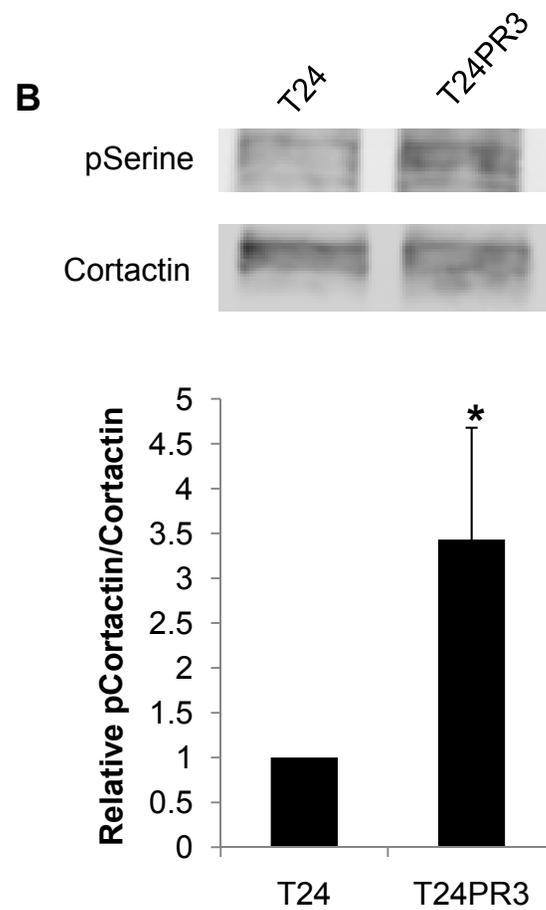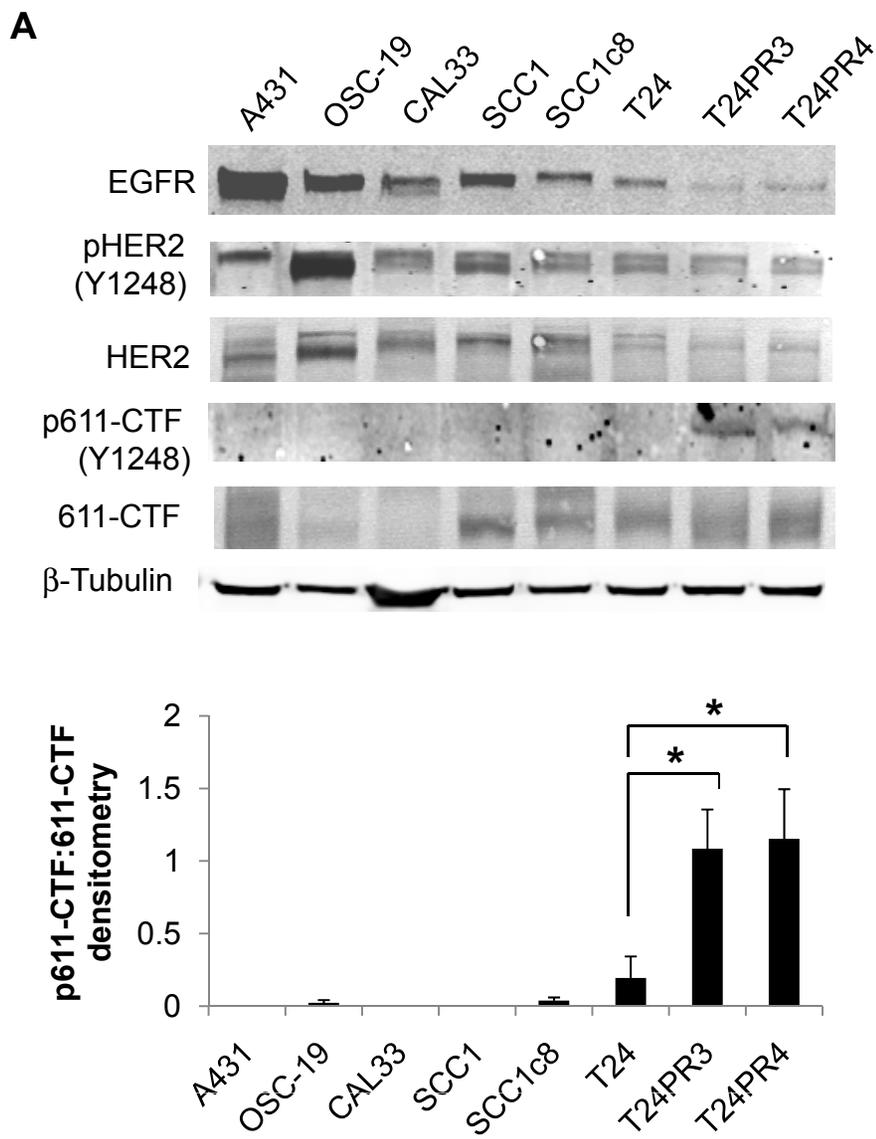
Clinical Cancer Research

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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