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Activation of ERBB2 Signaling Causes Resistance to the EGFR-Directed Therapeutic Antibody Cetuximab

Kimio Yonesaka,[1,2,3,4] Kreshnik Zejnullahu,[1,2] Isamu Okamoto,[3] Taroh Satoh,[3] Federico Cappuzzo,[5] John Souglakos,[6,7] Dalia Ercan,[1,2] Andrew Rogers,[1,2] Massimo Roncalli,[5] Masayuki Takeda,[3] Yasuhito Fujisaka,[3] Juliet Philips,[2] Toshio Shimizu,[3] Osamu Maenishi,[8] Yonggon Cho,[9] Jason Sun,[1,2] Annarita Destro,[5] Koichi Taira,[10] Koji Takeda,[10] Takafumi Okabe,[1,2] Jeffrey Swanson,[1,2] Hiroyuki Itoh,[8] Minoru Takada,[11] Eugene Lifshits,[12] Kiyotaka Okuno,[13] Jeffrey A. Engelman,[12] Ramesh A. Shivdasani,[2,14] Kazuto Nishio,[15] Masahiro Fukuoka,[4,11] Marileila Varella-Garcia,[9] Kazuhiko Nakagawa,[3]*† Pasi A. Jänne[1,2,14]*†

Cetuximab, an antibody directed against the epidermal growth factor receptor, is an effective clinical therapy for patients with colorectal, head and neck, and non–small cell lung cancer, particularly for those with *KRAS* and *BRAF* wild-type cancers. Treatment in all patients is limited eventually by the development of acquired resistance, but little is known about the underlying mechanism. Here, we show that activation of ERBB2 signaling in cell lines, either through *ERBB2* amplification or through heregulin up-regulation, leads to persistent extracellular signal–regulated kinase 1/2 signaling and consequently to cetuximab resistance. Inhibition of ERBB2 or disruption of ERBB2/ERBB3 heterodimerization restores cetuximab sensitivity in vitro and in vivo. A subset of colorectal cancer patients who exhibit either de novo or acquired resistance to cetuximab-based therapy has *ERBB2* amplification or high levels of circulating heregulin. Collectively, these findings identify two distinct resistance mechanisms, both of which promote aberrant ERBB2 signaling, that mediate cetuximab resistance. Moreover, these results suggest that ERBB2 inhibitors, in combination with cetuximab, represent a rational therapeutic strategy that should be assessed in patients with cetuximab-resistant cancers.

INTRODUCTION

Cetuximab, an epidermal growth factor receptor (EGFR)–directed antibody, is an effective treatment alone or in combination with chemotherapy for patients with colorectal cancer (CRC), head and neck squamous cell cancer (HNSCC), and non–small cell lung cancer (NSCLC) (*1*–*3*). Cetuximab functions by blocking ligand binding to the extracellular domain (ECD) of EGFR, thus preventing ligand-mediated EGFR signaling. In addition, cetuximab enhances receptor internalization and degradation and induces antibody-dependent cell-mediated cytotoxicity (*4*).

In patients with CRC, the initial clinical benefits of cetuximab are variable, and not all studies demonstrate a significant improvement in progression-free survival (PFS) or overall survival (OS) with cetuximab-based therapy (*5*, *6*). Prompted by these clinical observations and an increased understanding of EGFR signaling, several studies have evaluated the impact of oncogenic mutations in the EGFR signaling pathway on the efficacy of cetuximab in patients with metastatic CRC. Aberrant activation of downstream signaling pathways, especially those that result in activation of extracellular signal–regulated kinase 1/2 (ERK1/2) signaling, results in de novo clinical resistance to cetuximab-based therapy. These include mutations in *KRAS*, *BRAF*, and *NRAS* (*5*–*10*). Most, if not all, of the clinical benefits of cetuximab are limited to patients whose cancers do not harbor these oncogenic mutations (*11*). However, even among this molecularly enriched subset of patients, cetuximab is not uniformly clinically effective, suggesting that there are other, yet undefined, mechanisms of de novo cetuximab resistance (*5*–*9*). Identification of these additional resistance mechanisms may help further refine the subset of CRC patients likely to benefit from cetuximab or cetuximab-based combination therapies. In addition, although studies of genomic alterations in the EGFR signaling pathway can define the appropriate patient population to treat with a cetuximab-based regimen, all patients will ultimately develop resistance (acquired resistance) to cetuximab or other therapeutic EGFR antibodies. An understanding of acquired resistance mechanisms may help to identify effective therapies or guide the use of therapeutic combinations for patients who develop clinical cetuximab resistance. This strategy has been successful in studies of other molecular targeted therapies including EGFR kinase inhibitors (*12*).

To define additional mechanisms of de novo cetuximab resistance and to identify mechanisms of acquired cetuximab resistance, we generated and studied a series of cetuximab-resistant cell lines in vitro and in vivo. We combined our findings with studies of tumor specimens from cetuximab-treated CRC patients.

[1]Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. [3]Department of Medical Oncology, Kinki University School of Medicine, Osaka, Japan. [4]Department of Medical Oncology, Izumi Municipal Hospital, Osaka, Japan. [5]Istituto Clinico Humanitas, Rozzano, Italy. [6]Laboratory of Tumor Biology, Medical School, University of Crete, Heraklion, Greece. [7]University Hospital of Heraklion, Heraklion, Greece. [8]Department of Pathology, Kinki University School of Medicine, Osaka, Japan. [9]University of Colorado Cancer Center, Aurora, CO, USA. [10]Department of Clinical Oncology, Osaka City General Hospital, Osaka, Japan. [11]Department of Medical Oncology, Kinki University School of Medicine, Sakai Hospital, Osaka, Japan. [12]Massachusetts General Hospital Cancer Center, Boston, MA, USA. [13]Department of Surgery, Kinki University School of Medicine, Osaka, Japan. [14]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA. [15]Department of Genome Biology, Kinki University School of Medicine, Osaka, Japan.
*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: nakagawa@med.kindai.ac.jp (K.N.); pjanne@partners.org (P.A.J.)

RESULTS

ERBB2 amplification mediates cetuximab resistance

We first generated cetuximab-resistant HCC827 cells using previously described methods (*12*, *13*). We exposed cetuximab-sensitive HCC827 cells to increasing drug concentrations starting at 100 ng/ml, which is below the IC_{50} (median inhibitory concentration), until they were able to proliferate freely in cetuximab (100 μg/ml), similar to the maximal serum concentration observed in phase I studies (*14*, *15*). Four independent cetuximab-resistant clones were confirmed to have lost drug sensitivity (Fig. 1A). Unlike in the parental HCC827 cells, cetuximab did not fully inhibit phospho-ERK1/2 (pERK1/2) (Fig. 1B). However, the resistant HCC827 cells remained sensitive to the EGFR kinase inhibitor gefitinib (fig. S1A), which inhibited AKT and ERK1/2 phosphorylation and resulted in apoptosis (Fig. 1B and fig. S1B). Cetuximab treatment of HCC827 cells induced G_1-S arrest, consistent with down-regulation of pERK1/2, rather than phospho-AKT (pAKT), and lack of apoptosis (Fig. 1B and fig. S1B). F1

Genome-wide copy number analyses comparing HCC827 cetuximab-resistant cells with parental HCC827 cells (*12*, *16*) revealed a few small

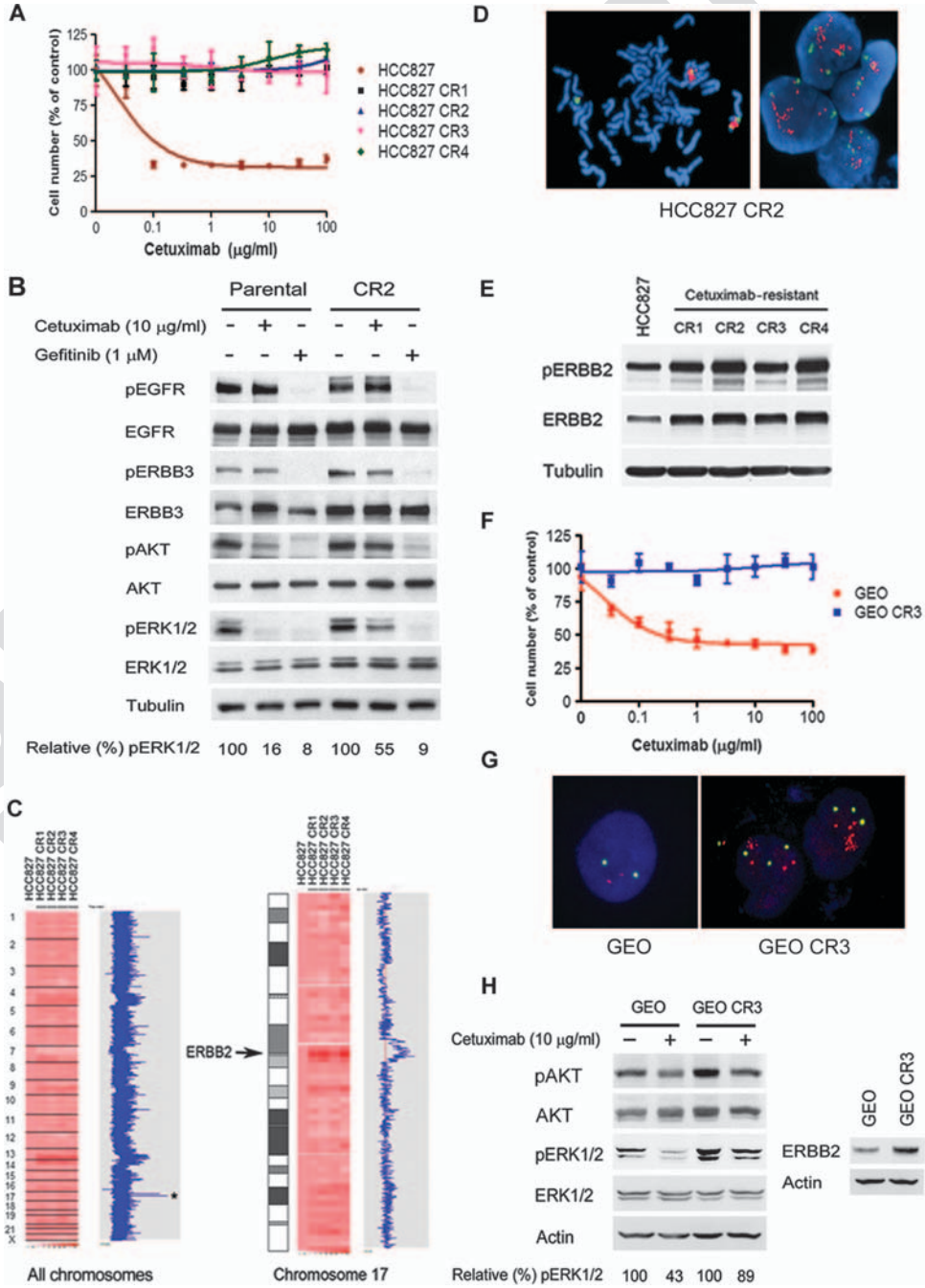

Fig. 1. Cetuximab-resistant NSCLC and CRC cells maintain ERK1/2 signaling and contain an *ERBB2* amplification. (**A**) Parental and resistant HCC827 cetuximab-resistant (CR) cells were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**B**) Parental HCC827 and CR2 cells were treated with cetuximab (10 μg/ml) or gefitinib (1 μM) for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (**C**) Amplification on chromosome 17 encompassing the *ERBB2* locus (asterisk, HCC827 cetuximab-resistant cells). The HCC827 cetuximab-resistant clones (right) were compared with parental HCC827 cells (first column). The blue curve on the right indicates degree of amplification of each SNP from 0 (left) to 8 (right). Left, genome-wide view; right, chromosome 17. (**D**) Metaphase (left) and interphase (right) FISH on HCC827 CR2 cells using ERBB2 (red) and CEP17 (green) probes. The HER2/CEP17 ratio was 4.7. (**E**) Expression of pERBB2 and ERBB2 in HCC827 and cetuximab-resistant cells. Cell extracts were immunoblotted to detect the indicated proteins. (**F**) Parental and resistant GEO CR3 cells were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**G**) Interphase FISH on GEO and GEO CR3 cells using ERBB2 (red) and CEP17 (green) probes. HER2/CEP17 ratio >2 was observed in 50% of GEO CR3 cells. (**H**) (Left) Parental GEO and CR3 cells were treated with cetuximab (10 μg/ml) for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (Right) Expression of ERBB2 in GEO and GEO CR3 cells.

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changes and a larger region of copy number gain on chromosome 17 (Fig. 1C), encompassing the *ERBB2* oncogene (Fig. 1C). Amplification in *ERBB2* was confirmed with fluorescence in situ hybridization (FISH; Fig. 1D), and the HCC827 cetuximab-resistant cells expressed higher levels of both total ERBB2 and phospho-ERBB2 (pERBB2) than the parental HCC827 cells (Fig. 1E).

HCC827 cells are an NSCLC cell line. Thus, we also determined whether *ERBB2* amplification also occurred in CRC, where cetuximab is in widespread clinical use, as a result of cetuximab exposure. We generated cetuximab-resistant clones of the GEO CRC cell line (Fig. 1F) and isolated seven independent resistant clones (fig. S1C). Three of the seven clones (CR3, CR7, and CR9) harbored evidence of *ERBB2* amplification (Fig. 1G and fig. S1C). Similar to the HCC827 cetuximab-resistant cells, the GEO CR3 cells expressed increased levels of ERBB2, and cetuximab did not effectively down-regulate pERK1/2 in these cells (Fig. 1H).

To determine whether ERBB2 plays a causal role in cetuximab resistance, we depleted *ERBB2* in the HCC827 cetuximab-resistant cells using an ERBB2-specific short hairpin RNA (shRNA), which restored both cetuximab sensitivity and its ability to down-regulate pERK1/2 (Fig. 2A). Furthermore, the combination of an ERBB2 antibody, trastuzumab, with cetuximab inhibited the growth of HCC827 CR2 (Fig. 2B) and GEO CR3 cells (Fig. 2C) compared to either agent alone. Treatment with the ERBB2 kinase inhibitor lapatinib restored sensitivity of HCC827 cetuximab-resistant cells to cetuximab (fig. S4A), and cetuximab was able to inhibit pERK1/2 in the presence of lapatinib (fig. S4B). Because lapatinib also inhibits EGFR, we introduced either a wild-type or a kinase-dead (K753M) *ERBB2* into HCC827 cells (Fig. 2D) to formally determine the requirement for ERBB2 kinase activity in mediating cetuximab resistance. ERBB2 K753M did not cause resistance to cetuximab (Fig. 2D), and cetuximab still inhibited ERK1/2 signaling in these cells (Fig. 2E). Collectively, these findings suggest that *ERBB2* amplification is the principal mechanism of resistance to cetuximab in both NSCLC and CRC cells and that inhibition of ERBB2, in conjunction with cetuximab, represents a potential treatment strategy for patients with acquired cetuximab resistance.

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ERBB2 amplification activates ERK1/2 signaling to mediate cetuximab resistance

To further evaluate whether ERBB2 could confer resistance in other cetuximab-sensitive cells, we used the HNSCC cell line HN11 and the NSCLC cell line H1648, both cetuximab-sensitive in vitro (*17*). Introduction of *ERBB2* to the cells conferred resistance to cetuximab in HCC827, HN11, and H1648 cells (Fig. 2F and fig. S2). In addition, cetuximab was unable to down-regulate pERK1/2 in either HCC827 or HN11 cells overexpressing ERBB2, in contrast to control green fluorescent protein (GFP)–infected cells (Fig. 2G and fig. S3A). Because *ERBB2* amplification could potentially interfere with cetuximab activity in several different ways, we asked whether activation of ERK1/2 signaling alone was sufficient to phenocopy the effects of *ERBB2* amplification. To this end, we introduced *BRAF* V600E into HCC827 or HN11 cells and evaluated the effects of cetuximab. Both cell lines became resistant to cetuximab (Fig. 2H and fig. S3B), which no longer fully inhibited pERK1/2 (Fig. 2I and fig. S3C). *BRAF* V600E is associated with cetuximab resistance in preclinical models and in CRC patients (*9*). Furthermore, growth factor receptor binding protein 2 (GRB2), a known mediator of ERK1/2 signaling, coprecipitated with ERBB2 in HCC827 CR2 and HN11 cells overexpressing *ERBB2* (Fig. 2J) (*18*). Finally, both GEO and the cetuximab-resistant GEO CR3 cells were equally sensitive to the MEK (mitogen-activated or extracellular signal–regulated protein kinase kinase) inhibitor AZD6244 (fig. S3D). ERBB2 did not inhibit cetuximab binding to EGFR in HCC827 cetuximab-resistant or HN11 ERBB2 cells, nor did it interfere with cetuximab-mediated internalization of EGFR. Collectively, these findings suggest that the principal mechanism by which ERBB2 causes cetuximab resistance is by activating ERK1/2 signaling.

Heregulin mediates resistance to cetuximab in models without evidence of ERBB2 amplification

To determine whether mechanisms other than *ERBB2* amplification could cause cetuximab resistance, we studied a cetuximab-resistant version of A431 cells (Fig. 3A and fig. S5A), which expressed increased levels of pERBB2 and pERBB3 but do not harbor increased total levels of ERBB2 or evidence of an *ERBB2* amplification (Fig. 3B). We hypothesized that these observations may be due to differences in ligands that activate ERBB2/ERBB3 signaling. A431CR cells produced an about 2.5-fold greater concentration of heregulin, measured in an enzyme-linked immunosorbent assay (ELISA), in cell culture medium than did the parental A431 cells (Fig. 3C); this was confirmed by Western blotting (fig. S5B). In the presence of heregulin, ERBB3 preferentially dimerizes with ERBB2 and consequently phosphorylates both ERBB proteins (*19*). Addition of heregulin to A431 cells led to dose-dependent increases in both pERBB2 and pERBB3 (fig. S5C). Furthermore, immunoprecipitation with an anti-ERBB2 antibody showed that in A431CR cells, there was increased association of ERBB2 with ERBB3 compared to the parental A431 cells (Fig. 3D). To examine whether heregulin loss could restore sensitivity to cetuximab in A431CR cells, we depleted heregulin using specific small interfering RNAs (siRNAs) in A431CR cells. Quantitative polymerase chain reaction (qPCR) demonstrated reduced heregulin expression, and immunoblotting revealed lower phosphorylation of both ERBB3 and AKT, a known mediator of ERBB3 signaling (fig. S5D) (*20*). Consistent with these findings, the cells demonstrated greater sensitivity to cetuximab ($P = 0.0007$, t test) (Fig. 3E). We then examined whether exogenous heregulin by itself could lead to resistance in cetuximab-sensitive cell lines. In A431 and the GEO and DiFi CRC cell lines, exogenous heregulin resulted in dose-dependent decreases in cetuximab sensitivity (Fig. 3F and fig. S6A). In the absence of heregulin, cetuximab readily reduced pERK1/2 in all cell lines (Fig. 3G and fig. S6B), whereas in the presence of heregulin, cetuximab had minimal or no effect on ERK1/2 phosphorylation. Heregulin treatment led to ERBB2 and ERBB3 phosphorylation in all three cell lines (Fig. 3G and fig. S6B).

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Inhibition of ERBB2 signaling restores cetuximab sensitivity in cells with heregulin-mediated cetuximab resistance

Our studies suggest that ERBB2 activation, and consequently cetuximab resistance, is a result of a heregulin autocrine loop in A431CR cells. To evaluate whether ERBB2 inhibition could represent a potential therapy in such cancers, we evaluated the effects of ERBB2 inhibition on cetuximab sensitivity using several complementary approaches. *ERBB2* depletion with an ERBB2-specific siRNA resulted in increased sensitivity to cetuximab (Fig. 4A). Furthermore, both A431 and A431 cetuximab-resistant cells were equally sensitive to the EGFR/ERBB2 dual kinase inhibitor lapatinib (Fig. 4B). We also treated A431CR cells with pertuzumab, an antibody that disrupts ERBB2/ERBB3 dimerization,

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alone and combined with cetuximab (*21*). Neither antibody alone significantly inhibited cell proliferation, whereas the combination of both did (Fig. 4C). Immunoblotting demonstrated that cetuximab was able to down-regulate pERK1/2 in the presence of pertuzumab in A431CR cells, whereas ERK1/2 remained persistently phosphorylated in the absence of pertuzumab (Fig. 4D).

ERBB2 amplification and increased heregulin mediate cetuximab resistance in vivo

Because EGFR-directed antibodies, including cetuximab, have several potential mechanisms of action, not all of which may be apparent in cultured cells, we further evaluated cetuximab resistance in vivo. Both cetuximab and gefitinib effectively inhibited xenografts generated from

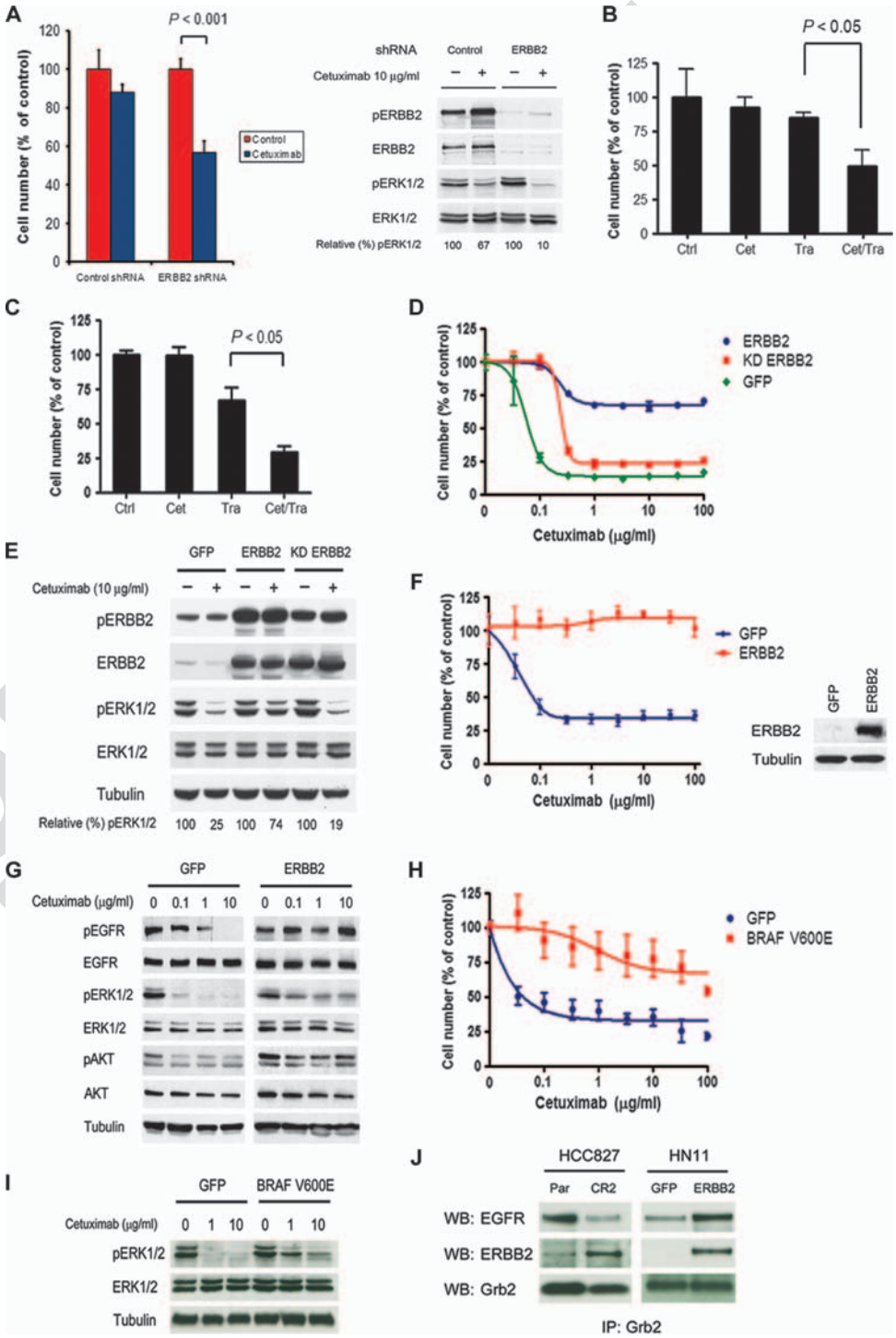

Fig. 2. Inhibition of ERBB2 restores cetuximab sensitivity in cetuximab-resistant cancer cell lines. (**A**) Depletion of *ERBB2* by an ERBB2-specific shRNA restores sensitivity to cetuximab. Control and *ERBB2* shRNA-treated HCC827 CR2 cells were treated with cetuximab (10 μg/ml), and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. Cell extracts were immunoblotted to detect the indicated proteins. (**B**) HCC827 CR2 cells were treated with cetuximab (Cet) (10 μg/ml) or trastuzumab (Tra) (10 μg/ml) alone or with both agents. Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls (Ctrl). (**C**) GEO CR3 cells were treated with cetuximab (10 μg/ml) or trastuzumab (10 μg/ml) alone or with both agents. Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. (**D**) HCC827 cells expressing GFP, ERBB2, or kinase-dead (KD) ERBB2 were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**E**) The indicated cell lines from (D) were untreated or treated with cetuximab (10 μg/ml) for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (**F**) HN11 cells expressing GFP or ERBB2 were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**G**) HN11 GFP and HN11 ERBB2 cells were treated with the indicated concentrations of cetuximab for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (**H**) HN11 cells expressing GFP or BRAFV600E were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**I**) Cells from (H) were treated with the indicated concentrations of cetuximab for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. (**J**) GRB2 coprecipitates with ERBB2 in HCC827 CR2 and HN11 ERBB2 cells. Cell extracts were immunoprecipitated with an anti-Grb2 antibody. The precipitated proteins were determined by immunoblotting with the indicated antibodies.

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F5 GFP-infected HCC827 cells (Fig. 5A), whereas only gefitinib inhibited the growth of HCC827 *ERBB2* xenografts. These tumors were resistant to cetuximab (Fig. 5A), similar to our in vitro observations (fig. S1A). Consistent with its effects on tumor growth, cetuximab treatment led to inhibition of pEGFR and down-regulation of total EGFR in the HCC827 GFP mice (Fig. 5B). In contrast, this was not observed, even after 2 weeks of treatment, in the HCC827 *ERBB2* tumors. Furthermore, ERBB2 coprecipitated with EGFR in the HCC827 ERBB2 tumors, suggesting formation of EGFR/ERBB2 heterodimers in these cetuximab-resistant tumors (Fig. 5B). We also evaluated the effects of cetuximab alone or in combination with pertuzumab in the A431 and A431CR cells (Fig. 5C). Cetuximab alone or in combination with pertuzumab effectively inhibited the growth of A431 xenografts (Fig. 5C). In contrast, only the combination of cetuximab and pertuzumab led to regression of A431CR xenografts (Fig. 5C), consistent with our in vitro findings (Fig. 4C).

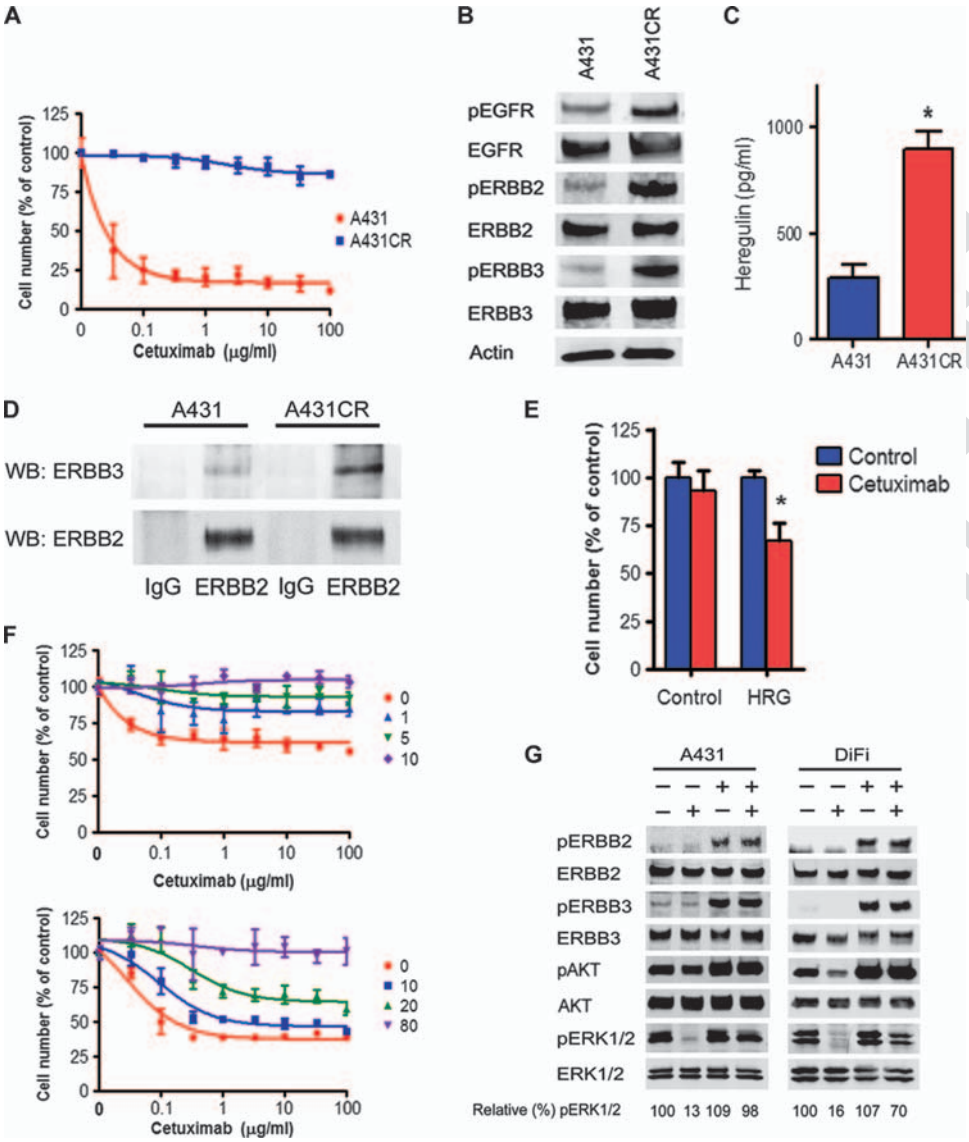

Fig. 3. Heregulin causes resistance to cetuximab. (**A**) Parental and cetuximab-resistant A431 cells were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**B**) A431 cetuximab-resistant cells have increased ERBB2 and ERBB3 phosphorylation. Cell extracts were immunoblotted to detect the indicated proteins. (**C**) Heregulin in cell culture medium was detected by ELISA from A431 and A431CR cells. *P = 0.0021, t test. (**D**) A431 and A431CR cell lysates were immunoprecipitated with anti-ERBB2 antibody. ERBB2 and ERBB3 were detected by immunoblotting. (**E**) Control or heregulin (HRG) siRNAs were transfected into A431CR cells, and cells were treated with cetuximab (100 μg/ml). The percentage of viable cells is shown (mean ± SD) relative to untreated control. *P = 0.0007 compared to control, t test. (**F**) A431 and DiFi cells were treated with cetuximab at the indicated concentrations in the presence of heregulin at the indicated concentrations (ng/ml). Viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. (**G**) A431 and DiFi cells were treated with cetuximab (10 μg/ml) alone, heregulin alone (10 ng/ml for A431; 20 ng/ml for DiFi), or the combination. Cells were lysed, and the indicated proteins were detected by immunoblotting.

ERBB2 amplification and increased heregulin are associated with de novo and acquired resistance in cetuximab-treated CRC patients

On the basis of our in vitro and in vivo findings demonstrating a role for both *ERBB2* amplification and heregulin in causing cetuximab resistance, we sought to determine whether these mechanisms also mediate clinical cetuximab resistance. These studies focused on CRC patients because cetuximab is in widespread clinical use in these patients and because our in vitro studies demonstrated that two CRC cell lines, GEO and DiFi, can develop cetuximab resistance through activation of ERBB2 signaling (*1*). We studied *ERBB2* amplification and heregulin as possible mediators of both de novo and acquired cetuximab resistance. Although our preclinical studies focused on mechanisms of acquired cetuximab resistance, we evaluated tumor and blood specimens from cetuximab-treated patients with either de novo or acquired resistance because acquired resistance mechanisms also cause de novo drug resistance as demonstrated for EGFR kinase inhibitors in NSCLC (*22*). We evaluated the clinical impact of de novo *ERBB2* amplification in a cohort of 233 CRC patients (*ERBB2* nonamplified, n = 220; *ERBB2*-amplified, n = 13) who had been treated with cetuximab alone or in combination with chemotherapy (table S1). The median PFS was longer for patients without *ERBB2* amplification (*ERBB2* nonamplified, 149 days; *ERBB2*-amplified, 89 days) (fig. S7). The median OS was significantly longer (*ERBB2* nonamplified, 515 days; *ERBB2*-amplified, 307 days) for patients without evidence of *ERBB2* amplification (P = 0.0013, log-rank test) compared to patients with *ERBB2*-amplified cancers (Fig. 6A). These findings F6

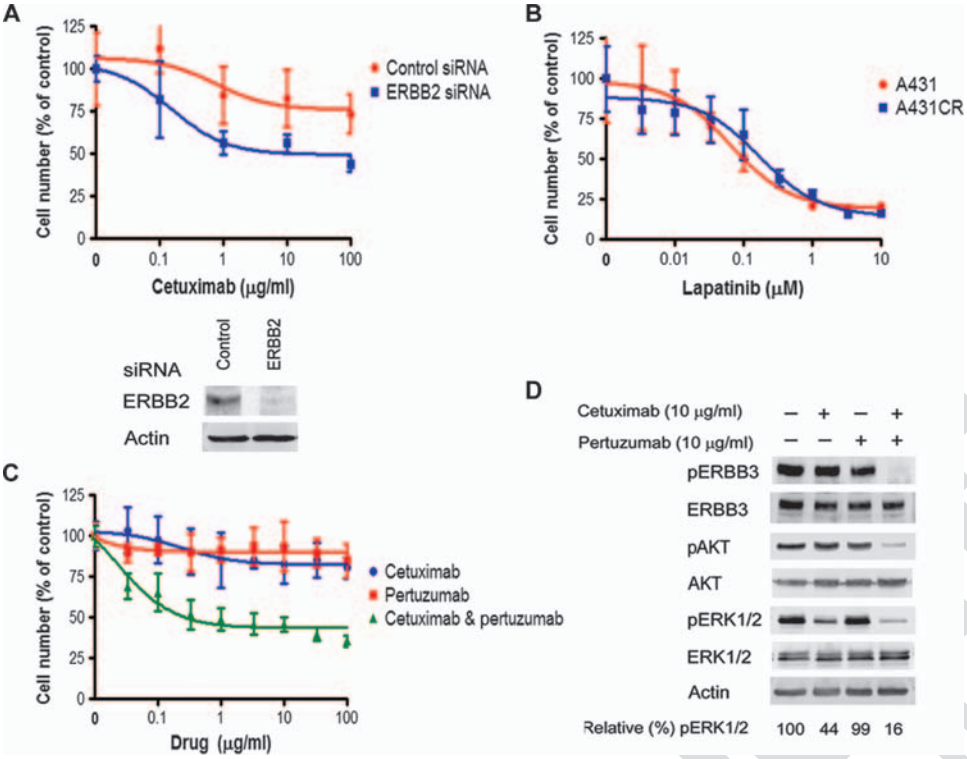

Fig. 4. ERBB2 inhibition restores cetuximab sensitivity in A431 cetuximab-resistant cells. (**A**) Cells transfected with control or ERBB2 siRNA were treated with the indicated concentrations of cetuximab. Viable cells were measured after 72 hours of treatment and plotted (mean ± SD) relative to untreated controls. ERBB2 expression was detected by immunoblotting. (**B**) A431 and A431 cetuximab-resistant cells are equally sensitive to lapatinib. (**C**) A431CR cells were treated with cetuximab alone, pertuzumab alone, or a combination of both drugs at the indicated concentrations, and viable cells were measured (mean ± SD) after 6 days of treatment. (**D**) A431CR cells were exposed to cetuximab alone (10 μg/ml), pertuzumab alone (10 μg/ml), or a combination of both drugs for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins.

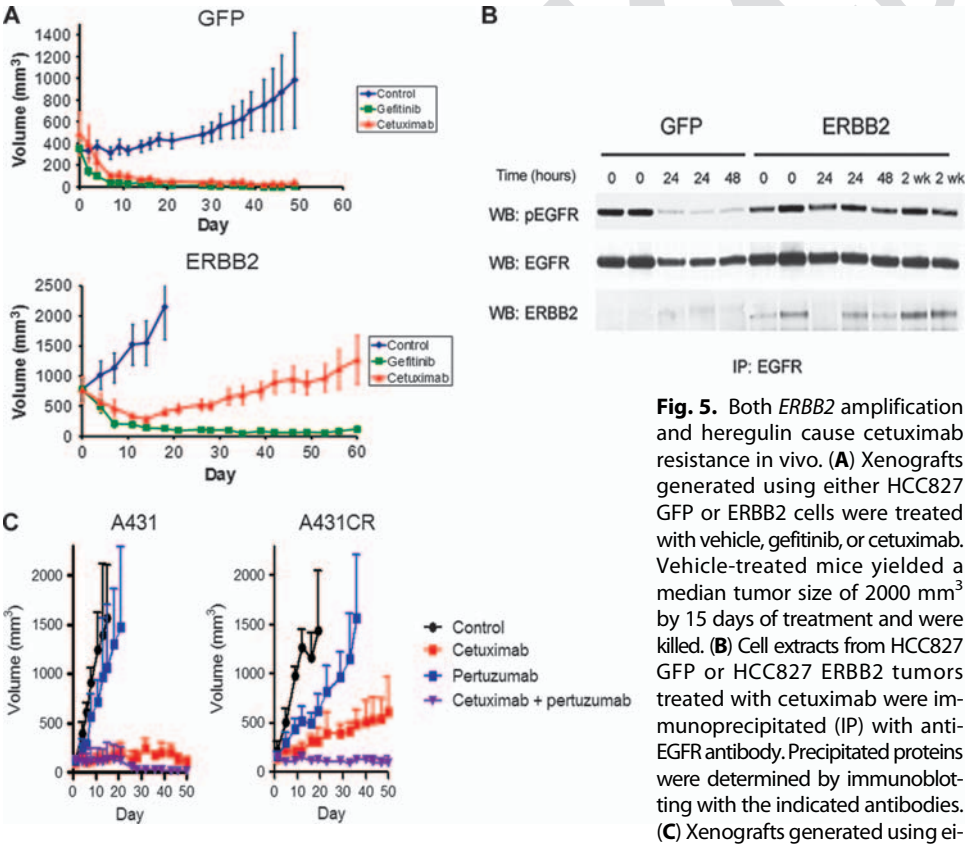

Fig. 5. Both *ERBB2* amplification and heregulin cause cetuximab resistance in vivo. (**A**) Xenografts generated using either HCC827 GFP or ERBB2 cells were treated with vehicle, gefitinib, or cetuximab. Vehicle-treated mice yielded a median tumor size of 2000 mm^3 by 15 days of treatment and were killed. (**B**) Cell extracts from HCC827 GFP or HCC827 ERBB2 tumors treated with cetuximab were immunoprecipitated (IP) with anti-EGFR antibody. Precipitated proteins were determined by immunoblotting with the indicated antibodies. (**C**) Xenografts generated using either A431 or A431 cetuximab-resistant cells were treated with vehicle, cetuximab alone, pertuzumab alone, or a combination of cetuximab and pertuzumab.

were similar when only patients with *KRAS* wild-type tumors were evaluated (Fig. 6A).

To assess a role for *ERBB2* amplification in acquired cetuximab resistance in patient tumors, we evaluated tumor specimens, obtained before and after cetuximab treatment, from two CRC patients who developed clinical cetuximab resistance. In both cases, there were substantially more *ERBB2*-amplified tumor cells in the posttreatment tumors compared to the pretreatment tumors (Fig. 6B and fig. S8A). In a separate cohort of nine patients, we used circulating serum levels of the ERBB2/HER2 ECD as a noninvasive surrogate measure of changes in tumor *ERBB2* after cetuximab treatment (fig. S8B and table S2) (*23*, *24*). In two of nine (22%) patients, both of whom previously had a partial clinical response to cetuximab-based therapy, serum HER2 ECD levels were substantially higher at the time of disease progression than before treatment (fig. S8B).

We also studied the relationship of heregulin to de novo cetuximab resistance in a separate cohort of 70 CRC patients treated with cetuximab-based therapy (table S3). Heregulin levels were evaluated with an ELISA in plasma samples obtained at baseline, before cetuximab exposure. Heregulin concentrations in plasma ranged widely (median, 1622.5 pg/ml; range, 0 to 18,045 pg/ml; Fig. 6C) but were significantly ($P < 0.0001$, t test) lower (Fig. 6C)

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in patients who had a partial response (PR) to cetuximab-based therapy (n = 16) than in those who had either stable disease (SD) or progressive disease (PD; n = 49). The same was true (P < 0.0001, t test) when the comparison was made only in patients with *KRAS* wild-type cancers (Fig. 6C). Because plasma heregulin may not fully reflect tumor heregulin concentrations, we asked whether tumor heregulin expression correlated with cetuximab efficacy. We isolated RNA from pretreatment tumor specimens in a subset of 44 of 70 (63%) patients, in whom tumor tissue was available, performed qPCR for heregulin (Fig. 6D), and correlated the findings with cetuximab efficacy. As in the ELISA studies, patients achieving a PR (n = 9) had significantly (P < 0.0001, t test) lower tumor heregulin expression compared to those with SD or PD (n = 35), whether we considered all patients or just those with *KRAS* wild-type (P = 0.0001, t test) cancers (Fig. 6D). We further divided the patients into two groups (low heregulin and high heregulin) based on the median plasma value (1622.5 pg/ml) and evaluated the relationship to PFS and OS. The low-heregulin group had significantly longer PFS (P = 0.004, log-rank test) and OS (P = 0.0014,

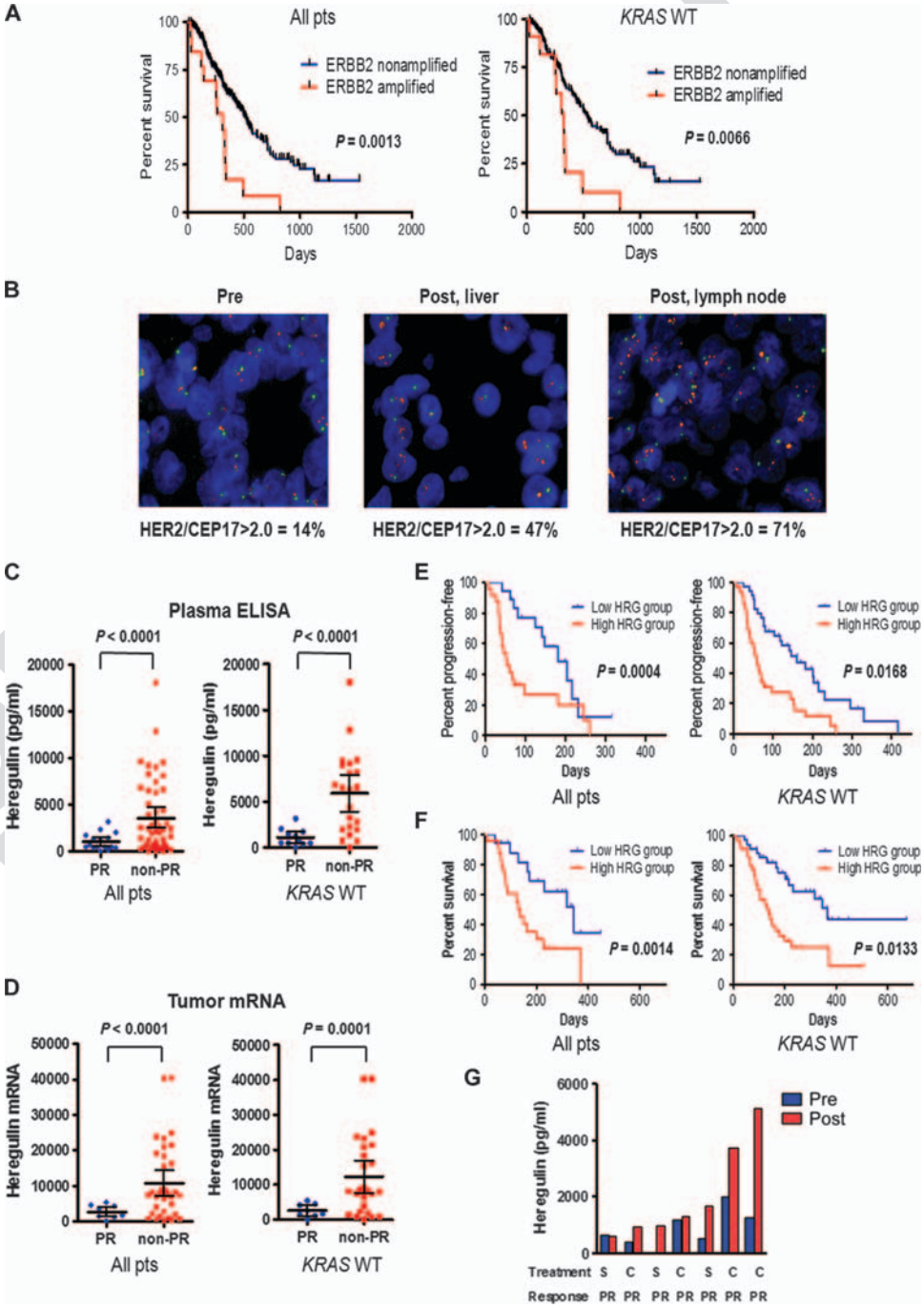

Fig. 6. Both *ERBB2* amplification and heregulin cause drug resistance in cetuximab-treated CRC patients. (**A**) (Left) OS for all CRC patients with (n = 13) and without (n = 220) *ERBB2* amplification treated with cetuximab-based therapy. Data for *KRAS* wild type (WT)–only patients (*ERBB2*-amplified, n = 11; *ERBB2* nonamplified, n = 171). Comparison based on log-rank test. (**B**) *ERBB2* FISH from a baseline primary tumor specimen (left) and after acquired cetuximab resistance in two independent drug-resistant specimens (right). The patient was initially treated with single-agent cetuximab and achieved a PR. ERBB2 (red) and CEP17 (green). (**C**) Scatter diagram of pretreatment heregulin concentration in plasma from all (n = 65) or *KRAS* wild type–only (n = 33) CRC patients achieving a PR and those not achieving a PR when treated with cetuximab-based therapy. Mean ± 95% CI is shown. (**D**) Scatter diagram of pretreatment heregulin mRNA expression in tumors from all (n = 44) or *KRAS* wild type–only (n = 34) CRC patients achieving a PR and those not achieving a PR when treated with cetuximab-based therapy. Mean ± 95% CI is shown. (**E**) (Left) PFS for all CRC patients treated with cetuximab-based therapy divided on the basis of low (n = 35) or high (n = 35) plasma expression. (Right) Data for *KRAS* wild type–only patients (low, n = 18; high, n = 24). Comparison based on log-rank test. (**F**) (Left) OS for all CRC patients treated with cetuximab-based therapy divided on the basis of low (n = 35) or high (n = 35) plasma expression. (Right) Data for *KRAS* wild type–only patients (low, n = 18; high, n = 24). Comparison based on log-rank test. (**G**) Comparisons of plasma levels of heregulin from CRC patients treated with cetuximab-based therapy before therapy and after the development of drug resistance. All patients achieved a PR. S, single-agent cetuximab; C, combination with irinotecan.

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log-rank test) compared to the high-heregulin group [median PFS, 161 versus 59 days; hazard ratio (HR), 0.36; 95% confidence interval (CI), 0.20 to 0.63; median OS, 366 versus 137 days; HR, 0.34; 95% CI, 0.18 to 0.66] when treated with cetuximab-based therapy (Fig. 6, E and F). This result was similar in *KRAS* wild-type patients (median PFS, 182 versus 52 days; HR, 0.41; 95% CI, 0.20 to 0.85; median OS, 345 versus 137 days; HR, 0.36; 95% CI, 0.16 to 0.80).

To evaluate the role of heregulin in acquired cetuximab resistance, we examined changes in serum heregulin levels after the development of drug resistance in seven patients, all of whom initially achieved a PR to cetuximab-based therapy (Fig. 6G). Compared to the pretreatment values, the posttreatment heregulin plasma concentrations were significantly higher ($P = 0.0313$, Wilcoxon signed-rank test) after the development of clinical cetuximab resistance (Fig. 6G). Collectively, these clinical studies further support our in vitro and in vivo studies and demonstrate that both *ERBB2* amplification and increased heregulin levels are associated with both de novo and acquired resistance to cetuximab-based therapy in CRC patients.

DISCUSSION

Studies of drug resistance mechanisms are critical for the development of effective cancer therapies. Mechanistic insights gained from studies of preclinical models and patient tumor specimens can be used to design new treatments or combination treatment strategies. This approach has led to the development of ABL kinase inhibitors for patients with imatinib-resistant chronic myeloid leukemia and the combination of EGFR and MET inhibitors for drug-resistant NSCLC (*12*, *25*, *26*).

Studies of drug resistance to EGFR inhibitors have focused on understanding resistance mechanisms to EGFR kinase inhibitors, and findings from the studies have been applied to develop the next generation of clinical trials (*12*, *27*, *28*). In contrast, there has been limited exploration of mechanisms of acquired resistance to EGFR-directed antibodies and none have been evaluated in cancer patients (*29*, *30*). Here, using a combination of resistant clones of cetuximab-sensitive cell lines coupled with analyses of cetuximab-treated CRC patients, we uncover aberrant ERBB2 signaling as a mediator of cetuximab resistance. We further demonstrate that aberrant ERBB2 signaling contributes to both de novo and acquired drug resistance in cetuximab-treated CRC patients.

Aberrant ERBB2 signaling (by *ERBB2* amplification or heregulin production) is an example of a resistance mechanism that leads to activation of a bypass signaling pathway. This is possible because ERBB2 is not the direct or indirect target of cetuximab. Both mechanisms of aberrant ERBB2 activation lead to persistent ERK1/2 signaling in the presence of cetuximab, thus preventing cetuximab-mediated growth inhibition (which is normally mediated by down-regulation of ERK1/2 signaling). In support of this hypothesis, both GEO and the *ERBB2*-amplified cetuximab-resistant GEO CR3 cells remain equally sensitive to the MEK inhibitor AZD6244. Notably, activation of EGFR signaling induces resistance to the ERBB2-directed therapeutic antibody trastuzumab in breast cancer cell lines in vitro and in vivo, suggesting a common mechanism for drug resistance to therapeutic antibodies in ERBB-driven cancers (*31*). *ERRB2* amplification is a unique mechanism of drug resistance in the case of cetuximab because *ERBB2*-amplified, cetuximab-resistant NSCLC cells remain sensitive to the EGFR kinase inhibitor gefitinib in vitro and in vivo (Fig. 5A and fig. S1A), likely because gefitinib, but not cetuximab, in addition to inhibiting EGFR, is also able to inhibit ERBB2 at clinically achievable concentrations (*32*).

Our findings are directly relevant to patients who develop acquired resistance to cetuximab-based therapy and may help guide subsequent treatment. Several agents that target ERBB2 signaling, including lapatinib and trastuzumab, are already approved and others, including irreversible ERBB2 kinase inhibitors and pertuzumab, are undergoing clinical development. Hence, the findings from the current study can be immediately used to design potential clinical therapies for CRC patients. Given the retrospective nature of the studies, these findings will need further clinical validation. The frequency and the relationship of *ERBB2* amplification to heregulin overexpression in cetuximab-resistant cancers need to be fully assessed in prospective studies. Our preclinical studies suggest that these are two independent means by which cancers can develop cetuximab resistance. However, whether *ERBB2* amplification and heregulin overexpression can occur together in the same drug-resistant tumor or tumor cells remains to be defined. It is intriguing that both increased levels of hepatocyte growth factor (HGF) and *MET* amplification have been observed in some EGFR kinase inhibitor–resistant NSCLCs (*16*).

Prospective clinical trials of cetuximab need to include evaluation of drug resistance mechanisms, including *ERBB2* amplification and heregulin measurements, at the time of disease progression. For patients with evidence of one of these drug resistance mechanisms, cetuximab combined with ERBB2-targeted therapy (for both mechanisms) or with an anti-ERBB3 antibody (heregulin only) should be further evaluated in clinical trials.

MATERIALS AND METHODS

Cell culture and reagents

The HCC827, H1648, HN11, GEO, A431, and DiFi cell lines have been previously characterized (*14*, *17*, *33*, *34*). Cetuximab and trastuzumab were purchased from the Dana-Farber Cancer Institute pharmacy. Gefitinib and lapatinib were purchased from American Custom Chemicals Corporation. Pertuzumab was provided by Roche Diagnostics. Cell proliferation and growth assays were performed with the MTS assay as described (*12*). All experimental points were a result of 6 to 12 replicates, and all experiments were repeated at least three times. The data were graphically displayed with GraphPad Prism version 5.0 for Windows (GraphPad Software).

Generation of drug-resistant cell lines

To generate drug-resistant cell lines, we exposed HCC827, GEO, and A431 cells to increasing concentrations of cetuximab similar to previously described methods (*12*, *13*). Individual clones from cetuximab-resistant cells were isolated and confirmed to be resistant.

Antibodies and Western blotting

Cells grown under the previously specified conditions were lysed in NP-40 buffer. Western blot analyses were conducted after separation by SDS–polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers' recommendations. Anti-pAKT (Ser^{473}), anti–total AKT, anti-pERBB2, anti-pERBB3, anti-ERBB2, and anti-EGFR antibodies were obtained from Cell Signaling Technology. The anti-heregulin

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antibody was purchased from NeoMarkers. The pEGFR (pY^{1068}), total ERK1/2, and pERK1/2 (pT^{185}/pY^{187}) antibodies were purchased from Invitrogen. Total ERBB3 antibody was purchased from Santa Cruz Biotechnology. The biotin-conjugated anti-EGFR antibody (ab24293) was obtained from Abcam. Relative pERK1/2 quantification was performed with the ImageJ 1.44 software.

Single-nucleotide polymorphism analyses

Single-nucleotide polymorphism (SNP) analyses were performed as described (*12*). Samples were processed for the Human Mapping 250K Sty SNP array according to the manufacturer's instructions. Comparison of gene copy number differences was performed with the dChip software according to previously established methods (*12*, *35*).

Site-directed mutagenesis

The *ERBB2* K753M (kinase dead) and the *BRAF* V600E mutations were introduced into *ERBB2* or *BRAF*, respectively, using site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis kit according to the manufacturer's instructions (*14*). All constructs were confirmed by DNA sequencing. The constructs were shuttled into the retroviral vector JP1540 with the BD CreatorPP System (BD Biosciences). Retroviral infections were carried out as described (*36*). Cells infected with a GFP expression vector were used as a control.

FISH analyses

Cell suspensions were dropped onto precleaned slides and air-dried. Three-day-old slides were analyzed with the dual-color FISH assay with the PathVysion DNA probe set. The slides were incubated in 70% acetic acid for 40 s, digested in 0.008% pepsin/0.01 M HC1 at 37°C for 5 min, fixed in 1% formaldehyde for 10 min, and dehydrated in an ethanol series. Formalin-fixed, paraffin-embedded (FFPE) tissue sections from CRC patients were subjected to a dual-color FISH assay with the PathVysion probe (LSI HER2 SO/CEP17 SG, Abbott Molecular). Initially, the slides were incubated from 2 hours to overnight at 56°C, deparaffinized in Citri-Solv, and washed in 100% ethanol for 10 min. The slides were sequentially incubated in 2× SSC at 75°C for 10 to 24 min, digested in proteinase K (0.25 mg/ml)/2× SSC at 45°C for 10 to 24 min, washed in 2× SSC for 5 min, and dehydrated in ethanol. The probe was applied according to the manufacturer's instructions to the selected hybridization area, which was covered with a glass coverslip and sealed with rubber cement. DNA denaturation was performed for 15 min at 85°C, and hybridization was allowed to occur at 37°C for 12 to 24 hours. Posthybridization washes were performed sequentially with 2× SSC/0.3% NP-40 (pH 7.0 to 7.5) at 73°C for 2 min and 2× SSC for 2 min, and dehydrated in ethanol. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (0.3 μg/ml) in Vectashield mounting medium (Vector Laboratories). Analysis was performed on an epifluorescence microscope with single interference filter sets for green [fluorescein isothiocyanate (FITC)], red (Texas red), blue (DAPI), dual (red/green), and triple (blue, red, green) band-pass filters.

shRNA and siRNA constructs and lentiviral infection

ERBB2 shRNA constructs cloned in pLKO.1 puro vector were described (*13*, *20*). A vector containing a nontargeting shRNA was used as a control. Lentivirus production, titrations, and infections were performed as in (*13*, *37*). The specific shRNA sequences are available upon request. The HRG1 (heregulin) siRNA was from Dharmacon (ON-TARGETplus SMARTpool #L-004608-01). HRG1 siRNA was a mixture of four sets of 21-nucleotide sense and antisense strands. ERBB2 siRNA was designed as follows: antisense, 5′-UGAGCUACCUGGAGGAUGUdTdT-3′. Control siRNA was nontargeting siRNA #1 (Dharmacon) and was used as a nonspecific control. For transfections, cells were plated at 50% confluence in six-well plates and incubated for 24 hours in RPMI 1640 supplemented with 0.1% fetal bovine serum (FBS). Cells were then treated with siRNAs mixed with Lipofectamine RNAiMax (Invitrogen).

Phospho-RTK array

Cells were lysed with NP-40 lysis buffer after incubation in RPMI 1640 supplemented with 0.1% FBS for 24 hours. Cell lysates were centrifuged at 14,000*g* for 5 min. Supernatants were transferred to and incubated with the Human Phospho-RTK Array (R&D Systems) according to the manufacturer's procedure.

Xenograft studies

The xenograft studies were performed with the HCC827 GFP, HCC827 ERBB2, A431, and A431 cetuximab-resistant cells as described (*38*). Cetuximab or pertuzumab was administered by intraperitoneal injection (cetuximab: 40 mg/kg, twice weekly; pertuzumab: 12 mg/kg, week 1 followed by 6 mg/kg weekly), and gefitinib (150 mg/kg per day) by oral gavage (*38*). The studies were performed in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee of the Beth Israel Deaconess Medical Center and the Kinki University.

Patients

Plasma and tumor specimens from CRC patients treated with cetuximab-based therapy were obtained from Istituto Clinico Humanitas (Rozzano, Italy), University Hospital of Heraklion (Heraklion, Greece), the Dana-Farber Cancer Institute/Brigham and Women's Hospital (Boston, MA), Kinki University Hospital (Osaka, Japan), Osaka City General Hospital (Osaka, Japan), Kinki University Sakai Hospital (Osaka, Japan), and the Kinki University Nara Hospital (Nara, Japan) under Institutional Review Board–approved studies. All patients provided written informed consent.

HER2 ECD measurements

Plasma specimens were obtained from nine CRC patients before cetuximab treatment and after development of acquired resistance to cetuximab. ERBB2/HER2 ECD was measured with an ELISA assay according to the manufacturer's recommended conditions (Siemens Healthcare Diagnostics). Only patients who developed PR or SD were included in these analyses. The studies were approved by the Institutional Review Board at the Kinki University.

KRAS sequencing

DNA was extracted from each tissue specimen with standard techniques. Codons 12 and 13 of exon 2 of *KRAS* were sequenced directly.

Quantitative heregulin PCR from cells and primary tumors

Total RNA was isolated from A431 and A431CR cells with the RNeasy Mini Kit (Qiagen), according to the manufacturer's specifications. The complementary DNA (cDNA) was synthesized with RT Enzyme Mix (Applied Biosystems) according to the manufacturer's specifications and was used for real-time PCR with SYBR Green (Cambrex Bio Science) to measure GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and heregulin.

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All tumor specimen samples were formalin-fixed and paraffin-embedded. Tumor RNA was isolated from FFPE tumors with the RNeasy FFPE kit (Qiagen). RNA was extracted form DNA digestion with DNaseI according to the manufacturer's protocol. Reverse transcription was performed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems) and was followed by quantitative reverse transcription–PCR (RT-PCR) with a Solaris qPCR GENE Expression Assays SYBR (Thermo Fischer Scientific) measured by ABI PRISM 7900HT (Applied Biosystems). Heregulin expression was measured in duplicate and normalized against the reference gene *GAPDH*.

Heregulin ELISA assay

Heregulin was measured in cell culture medium or human plasma with a sandwich ELISA (NRG1-β1 DuoSet) with methods as described (*17*). Cells were seeded in six-well plates at a concentration of 0.5×10^6 cells per well with RPMI 1640 supplemented with 10% FBS. After confluent growth, the medium was replaced with 5 ml of RPMI 1640 supplemented with 0.1% FBS. After a 48-hour incubation, cell culture medium was collected. Human plasma samples were obtained from CRC patients within a week before cetuximab treatment. All subjects provided written informed consent. In the case of patients who acquired resistance to cetuximab, samples were also obtained at the point of disease progression. After centrifugation at 15,000 rpm for 3 min, the supernatant was collected.

Statistical analysis

Statistical analysis was performed with the StatView statistical program (SAS Institute) to compare patient characteristics with responses to therapy. PFS and OS curves were generated with the Kaplan-Meier method, and differences based on *ERBB2* amplification and median heregulin plasma levels were evaluated with the log-rank test. All *P* values are two-sided.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/3/99/99ra86/DC1

Oligonucleotide sequences

Fig. S1. Cetuximab-resistant HCC827 and GEO cells.

Fig. S2. ERBB2 causes resistance to cetuximab in cetuximab-sensitive cells.

Fig. S3. ERBB2 maintains ERK1/2 signaling in the presence of cetuximab.

Fig. S4. Lapatinib restores sensitivity to cetuximab.

Fig. S5. Heregulin mediates resistance to cetuximab in A431 cells.

Fig. S6. Heregulin mediates resistance to cetuximab in the GEO cells.

Fig. S7. Progression-free survival for all CRC patients treated with cetuximab-based therapy.

Fig. S8. Increased *ERBB2* copy number is associated with acquired cetuximab resistance.

Table S1. Characteristics of colorectal cancer patients used to evaluate impact of *ERBB2* amplification.

Table S2. Clinical and treatment information on colorectal cancer patients used for plasma ERBB2 extracellular domain measurements.

Table S3. Characteristics of colorectal cancer patients used for plasma- and tumor-based studies of heregulin.

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Abstracts

One-sentence summary: Several cancers become resistant to cetuximab by activating a bypass signaling pathway and preventing cetuximab inhibition of ERK1/2-stimulated growth.

Editor's Summary:
Combating Resistance to an EGF Receptor Inhibitor

Many promising anticancer drugs are effective only for a limited time, because the tumor cells develop resistance. Cetuximab, directed against the epidermal growth factor receptor (EGFR), is no exception, and patients with colorectal, head and neck, or non–small cell lung cancer eventually cease to respond to the drug. Yonesaka and colleagues have determined that cetuximab-resistant cancer cells—both in culture and in patients—can up-regulate signaling through the ERBB2 growth factor receptor in several ways, permanently turning on extracellular signal–regulated kinase 1/2 (ERK1/2)–mediated growth, differentiation, and survival. They further show that interference with the ERBB2 pathway restores the ability of cetuximab to control these cancers, pointing to a promising resistance-fighting approach.

The authors generated clones of cetuximab-resistant non–small cell lung and colorectal cancer cell lines by exposing the cells to increasing concentration of the drug. In some of these resistant clones, the ERBB2 receptor oncogene was genetically amplified, resulting in activated ERK1/2 signaling. Down-regulation of ERBB2 with a small interfering RNA or antibody restored sensitivity. Other clones did not have amplified ERBB2 genes but did make excess heregulin, an activating ligand for the ERBB2 receptor. Heregulin depletion or ERBB2 inhibition restored cetuximab sensitivity.

After replicating these studies in xenografts in mice, the authors also looked for evidence that these resistance-associated alterations pertain to human tumors. In several groups of patients with colorectal cancer, they saw decreased survival or decreased sensitivity to cetuximab in those who exhibited amplified ERBB2 gene or higher heregulin concentrations. The concordance of their cellular data with patient experience improves confidence that concomitant treatment of certain lung, head and neck, or colorectal cancers with cetuximab and an anti-ERBB2 drug may prevent or delay the development of drug resistance. These studies add to other successes for this approach, which has also been used for analysis of other molecular targeted therapies, including EGFR kinase inhibitors.

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