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IGF2 is an actionable target that identifies a distinct subpopulation of colorectal cancer patients with marginal response to anti-EGFR therapies

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Abstract

Among patients with colorectal cancer who benefit from therapy targeted to the epidermal growth factor receptor (EGFR), stable disease (SD) occurs more frequently than massive regressions. Exploring the mechanisms of this incomplete sensitivity to devise more efficacious treatments will likely improve patients' outcomes. We tested therapies tailored around hypothesis-generating molecular features in patient-derived xenografts ("xenopatient"), which originated from 125 independent samples that did not harbor established resistance-conferring mutations. Samples from xenopatient that responded to cetuximab, an anti-EGFR agent, with disease stabilization displayed high levels of EGFR family ligands and receptors, indicating high EGFR pathway activity. Five of 21 SD models (23.8%) characterized by particularly high expression of EGFR and EGFR family members regressed after intensified EGFR blockade by cetuximab and a small-molecule inhibitor. In addition, a subset of cases in which enhanced EGFR inhibition was unproductive (6 of 16, 37.5%) exhibited marked overexpression of insulin-like growth factor 2 (IGF2). Enrichment of IGF2 overexpressors among cases with SD was demonstrated in the entire xenopatient collection and was confirmed in patients by mining clinical gene expression data sets. In functional studies, IGF2 overproduction attenuated the efficacy of cetuximab. Conversely, interception of IGF2-dependent signaling in IGF2-overexpressing xenopatient potentiated the effects of cetuximab. The clinical implementation of IGF inhibitors awaits reliable predictors of response, but the results of this study suggest rational combination therapies for colorectal cancer and provide evidence for IGF2 as a biomarker of reduced tumor sensitivity to anti-EGFR therapy and a determinant of response to combined IGF2/EGFR targeting.

INTRODUCTION

Monoclonal antibodies that target the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, achieve clinically meaningful rates of response, disease control, and survival in patients with chemorefractory metastatic colorectal cancer (mCRC) (1–5). Genetic alterations in *EGFR*, which are known to predict response to EGFR inhibitors, have not been consistently detected in CRC. However, a number of other genetic biomarkers robustly correlate with lack of sensitivity. All such resistance biomarkers trigger constitutive activation of signaling pathways that are parallel to or

downstream from EGFR and include mutations in *KRAS* and *NRAS* (exons 2 to 4), *BRAF* (exon 15), and *PIK3CA* (exon 20) (3–8), as well as gene amplification of *KRAS*, *HER2*, and *MET* (9–13). The assessment of *KRAS* mutational status in exon 2 is now widely deployed as a certified companion diagnostic to spare patients from futile treatment. It is foreseen that routine determination of additional alterations will soon enter clinical practice.

As expected, exclusion from anti-EGFR therapy of mCRC patients bearing resistance-conferring mutations has advanced (and will further advance) the identification of sensitive cases. However, the outlook for responders remains guarded, with only 2-month improvement in progression-free survival (PFS) and 6-month improvement in overall survival (OS) compared with chemotherapy (5). The conclusion from such data is that the impact of EGFR-targeted therapies in mCRC has been incremental rather than transformative, almost 10 years since their approval. One of the reasons for this shortfall is that the vast majority of mCRCs that prove to be sensitive to EGFR inhibition exhibit marginal responses, with impaired tumor growth and small reductions of tumor volumes prevailing over marked regressions (3, 4). This situation stands in contrast to that of other tumor types with genetically based sensitivity to EGFR-targeted agents. For example, in non-small cell lung carcinomas with *EGFR* gene mutations, EGFR inhibitors often induce considerable shrinkage (14, 15).

The aim of this study was to discover rational ways to shift the distribution of anti-EGFR responses from a preponderance of tumor growth inhibition to a higher frequency of overt tumor shrinkage in colorectal cancer, based on tumor molecular characteristics. To do this, we evaluated a large series of patient-derived xenografts from CRC liver metastases, which were profiled for several molecular parameters and concomitantly annotated for response to single-agent cetuximab and to combination therapies with other targeted agents in vivo in mouse cohorts (“xenopatients”) (10).

RESULTS

Response to cetuximab in “quadruple negative” mCRC xenopatients: A population study in 125 cases

Clinical studies have recently demonstrated that, in addition to the validated *KRAS* mutations in exon 2, additional *KRAS* mutations in exons 3 and 4, as well as *NRAS*, *BRAF*, and concurrent *PIK3CA* (exon 20) mutations, predict a lack of response in patients who receive anti-EGFR antibodies (5, 8). We obtained similar results in a series of 66 xenopatients wild type for exon 2 of *KRAS* (10). We decided to expand our investigation of cetuximab activity on a wider population scale, with a specific focus on quadruple negative cases that did not harbor resistance-conferring mutations in *KRAS*, *NRAS*, *BRAF*, or *PIK3CA*. We therefore assessed anti-EGFR response in an extended suite of xenopatients, for a total of 125 quadruple negative models (77 newly engrafted samples and 48 samples from our former collection) (10). In accordance with previously established procedures (10), we defined a “case” as one (or, more often, two) xenopatient lines derived from an individual patient specimen, with each line consisting of 12 mice (6 treated with placebo and 6 treated with cetuximab). To segregate response classes, we adopted a volume-based designation inspired by clinical standards: (i) regression [objective response (OR)] was classified as a reduction of at least 50% in the mean volume of tumors for each case, taking as reference the baseline (pretreatment) tumor volume; (ii) progressive disease (PD) was categorized as a volume increase of at least 35%; and, finally, (iii) intermediate responses (tumor volume changes between 35% increase and 50% reduction) were considered as stable disease (SD). On the basis of comparative calculations, these three-dimensional cutoffs were the closest approximations for the unidimensional RECIST (Response Evaluation Criteria in Solid Tumors) criteria used in patients, which refer to the longest diameter of target lesions.

Figure 1A depicts a waterfall plot of the effect of cetuximab at 3 weeks after the start of treatment, with xenopatient cases ranked by tumor volume changes relative to baseline: OR was observed in 31 cases (24.8%), SD in 60 cases (48%), and PD in 34 cases (27.2%). Because this set of xenopatients was chosen to include a genetically selected subpopulation free of resistance-conferring mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*, the distribution of response rates compared favorably with data obtained in less stringently selected series. Indeed, mCRC patients for whom only *KRAS* (exon 2) wild-type status was assessed have been shown to experience a 12.8 to 17% frequency of tumor

regression and a 34% frequency of SD (3, 4). Similarly, in our original collection of 66 xenopatient cases—which excluded *KRAS* (exon 2) mutations, but included all other mutant genotypes—OR occurred in 11 cases (16.7%) and SD in 27 cases (40.9%) (10).

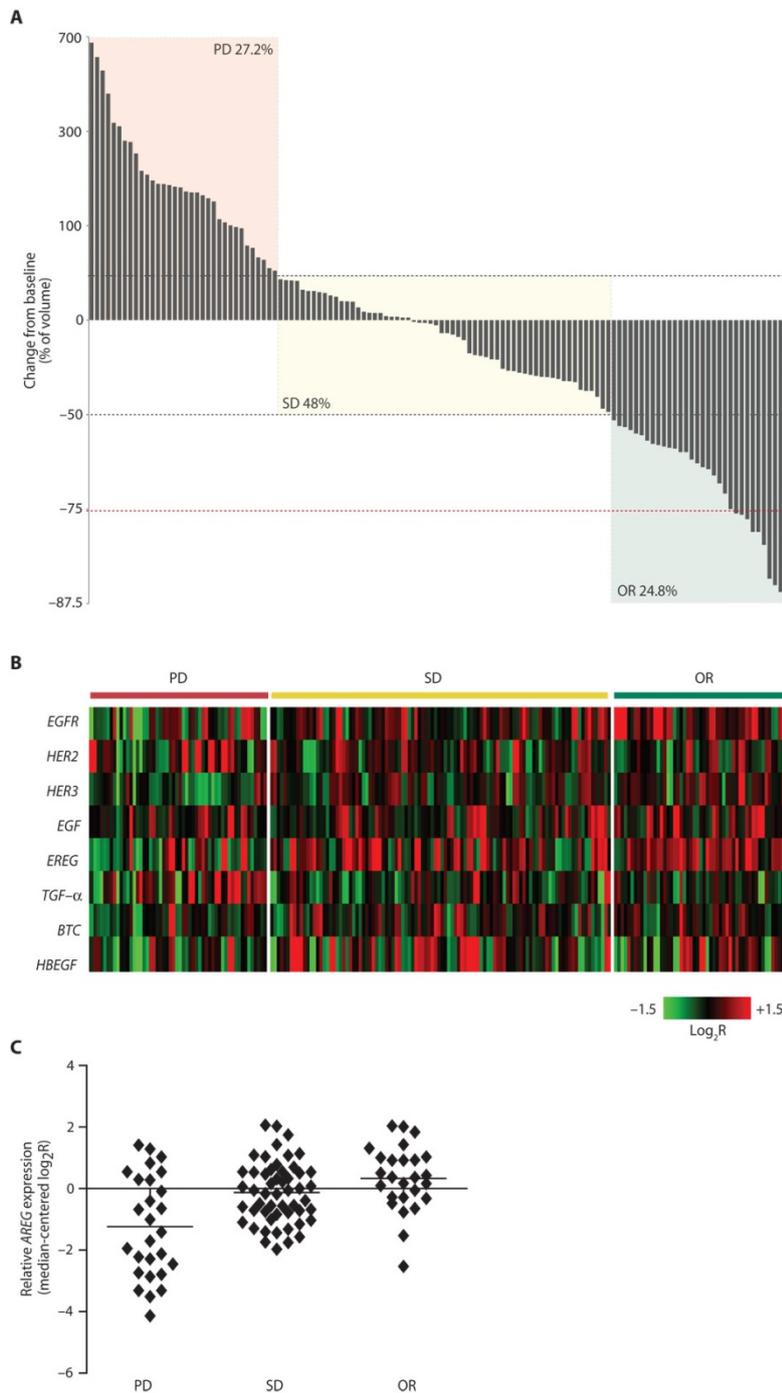


Fig. 1. Response to cetuximab and correlations with expression of EGFR family receptors and ligands.

(A) Waterfall plot of cetuximab response after 3 weeks of treatment, compared with tumor volume at baseline, in a population of 125 quadruple negative xenopatient cases. Dotted lines indicate the cutoff values for arbitrarily defined categories of therapy response: cases experiencing PD (tumor volume increase of at least 35%), SD (tumor volume changes between 35% increase and 50% reduction), or OR (tumor volume reduction of at least 50%) are shaded in light red, light yellow, and light aquamarine, respectively. Cases with OR higher than 75% tumor volume reduction (massive regressions) are separated by the red dotted line. (B) Heatmap depicting the gene expression levels of EGFR family receptors and ligands in 105 cases from the same collection, clustered according to cetuximab response. Gene expression was extracted from an Illumina beadchip array data set (table S1). Each case

was profiled in two samples from the same patient's tumor propagated in two different mice and is ranked according to the waterfall plot shown in (A). Gene expression levels, median-centered \log_2R : *EGFR*, PD -0.16 , SD 0.18 , OR 0.61 ; OR versus PD, $P = 2 \times 10^{-5}$ by two-tailed Student's *t* test; SD versus PD, $P = 0.013$. *HER2*, PD 0.37 , SD 0.11 , OR 0.00 ; OR versus PD, $P = 0.122$; SD versus PD, $P = 0.266$. *HER3*, PD -0.43 , SD 0.05 , OR 0.19 ; OR versus PD, $P = 2 \times 10^{-4}$; SD versus PD, $P = 0.002$. *EGF*, PD 0.19 , SD 0.17 , OR 0.31 ; OR versus PD, $P = 0.299$; SD versus PD, $P = 0.864$. Epiregulin (*EREG*), PD -0.09 , SD 0.34 , OR 0.55 ; OR versus PD, $P = 2 \times 10^{-6}$; SD versus PD, $P = 7 \times 10^{-4}$. Transforming growth factor- α (*TGF- α*), PD 0.17 , SD -0.06 , OR -0.09 ; OR versus PD, $P = 0.054$; SD versus PD, $P = 0.089$. β -Cellulin (*BTC*), PD -0.10 , SD 0.08 , OR 0.10 ; OR versus PD, $P = 0.031$; SD versus PD, $P = 0.040$. Heparin-binding EGF (*HBEGF*), PD -0.18 , SD 0.16 , OR 0.03 ; OR versus PD, $P = 0.158$; SD versus PD, $P = 0.008$. See also fig. S2 for graphic representation of expression data. (C) RT-qPCR analysis of amphiregulin (*AREG*) expression in the 105 cases shown in (B). Gene expression levels, median-centered \log_2R : PD -1.2 , SD -0.1 , OR 0.3 ; OR versus PD, $P = 5 \times 10^{-4}$; SD versus PD, $P = 0.011$.

Although inclusion of only quadruple negative tumors led to a higher frequency of treatment response, cases with SD were still predominant over regressions (Fig. 1A). Moreover, cases exhibiting overt tumor shrinkage [defined as a mass reduction of more than 75%, a cutoff arbitrarily set at a size equidistant between borderline regression (50%) and complete mass obliteration (100%)] remained a small minority (9 of 125, 7.2%) (Fig. 1A). These findings suggest that this refinement of genetic selection may not be sufficient to enrich for full responders. We therefore elected to exploit the merits of xenopatient—namely, their suitability for multiple exploratory therapies and the tumor molecular information associated with each xenopatient model—to test rational combinations as a means to improve the efficacy of single-agent cetuximab in SD cases.

Molecular correlations between expression of EGFR pathway components and response to cetuximab

Retrospective clinical trials have shown a correlation between gene copy number gain (and consequent overexpression) of *EGFR* or higher expression of two EGFR ligands, amphiregulin and epiregulin, and increased sensitivity to anti-EGFR treatment (16–21). We broadened these observations by extracting the transcript levels of EGFR family receptors with known activity in CRC (*EGFR*, *HER2*, and *HER3*) and EGFR cognate ligands (*EGF*, epiregulin, *TGF- α* , β -cellulin, and heparin-binding EGF) out of an Illumina beadchip array data set. Baseline gene expression information was available for 105 of the 125 quadruple negative models analyzed for cetuximab response; for each model, two independent xenografts were subjected to microarray analysis, for a total of 210 profiles (table S1). Because amphiregulin expression proved to be undetectable (likely due to probe failure), the same cases were profiled for amphiregulin expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR). We note that these surveys were performed on tumor samples after the first passage in vivo. In this condition, the human stroma was substituted by murine components, as demonstrated by lack of immunoreactivity for vimentin, a prototypical stromal marker, when using human-specific antibodies (fig. S1); therefore, this analysis covered only receptors and autocrine ligands expressed by cancer cells.

As expected, we observed a positive association between elevated expression of *EGFR*, epiregulin, and amphiregulin and OR to cetuximab (Fig. 1, B and C, fig. S2, and table S1). Again, in accordance with previous observations (10–12, 22), *HER2* was strongly overexpressed in a fraction of PDs, and the expression of *TGF- α* was increased in a portion of cases displaying resistance to treatment (Fig. 1B, fig. S2, and table S1). Extension of gene expression analysis to the other receptors and ligands revealed that the *HER3* receptor, β -cellulin, and heparin-binding EGF were overall more expressed in responders than in resistant cases (Fig. 1B, fig. S2, and table S1).

In addition to baseline profiles, on-treatment microarray data were also available for 14 cetuximab-sensitive models that had received the antibody for 72 hours or 6 weeks (table S2). This longitudinal analysis indicated progressive reduction (with statistical significance after 6 weeks) in the expression levels of *EGFR* ($P = 0.017$ relative to baseline expression), epiregulin ($P = 1 \times 10^{-4}$), and heparin-binding EGF ($P = 0.035$), and increased expression of *HER2* ($P = 0.002$) and *HER3* ($P = 0.013$) (fig. S3, A and B). This suggests gradual tumor adaptation to EGFR blockade by down-regulation of EGFR-dependent signals and up-regulation of *HER2/HER3*-based compensatory pathways.

Combination therapies against the EGFR pathway in xenopatient showing partial sensitivity to single-agent cetuximab

When compared with resistant cases, the baseline expression of EGFR, HER3, amphiregulin, epiregulin, β -cellulin, and heparin-binding EGF was higher not only in responders but also in SDs (Fig. 1, B and C, fig. S2, and table S1); heparin-binding EGF was even more expressed in SDs than in ORs (Fig. 1B, fig. S2, and table S1). Increased representation of EGFR, HER3, and EGFR ligands not only in ORs but also in SDs suggests high EGFR pathway activity in this latter subpopulation, which may sustain a certain degree of functional dependency on EGFR signals.

On the basis of these clues, we explored whether potentiated inactivation of EGFR was sufficient to induce massive regression (more than 75%) in those tumors that responded to cetuximab alone with only growth inhibition or limited shrinkage. Specifically, we randomly extracted from our entire collection of quadruple negative xenopatient 21 SD cases (fig. S4) and comparatively tested the effects of cetuximab alone versus a combination of cetuximab plus lapatinib, a dual EGFR/HER2 small-molecule inhibitor (combo1). After 4 weeks of treatment with combo1, five cases showed more than 75% tumor volume reduction (Fig. 2A), which was not achieved by single-agent treatment with cetuximab (table S3). Figure 2B shows the response to individual inhibitors or combo1 over time in three representative cases. Lapatinib alone was overall poorly effective, irrespective of the final outcome of the combination therapy. In cases in which combo1 was advantageous, regression occurred rapidly and proceeded steadily until almost complete eradication of the tumor mass (M079). In other instances, tumor growth kinetics were affected similarly by cetuximab alone and cetuximab plus lapatinib, with cases experiencing either long-lasting (M304) or short-lived (M241) disease stabilization.

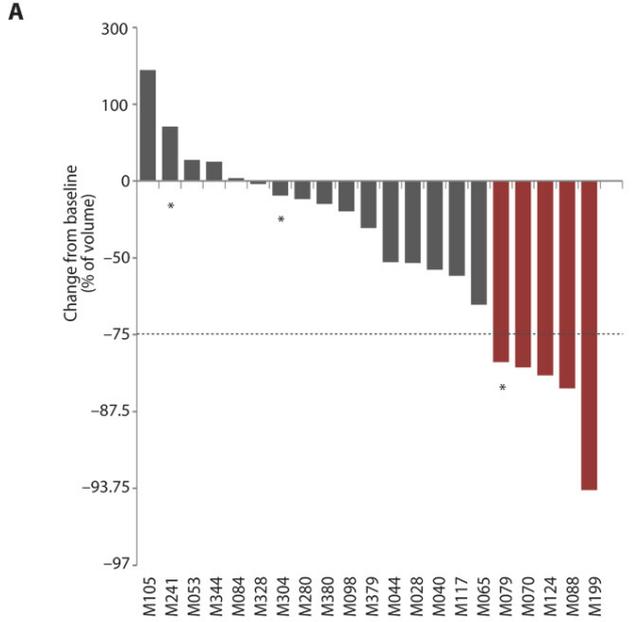
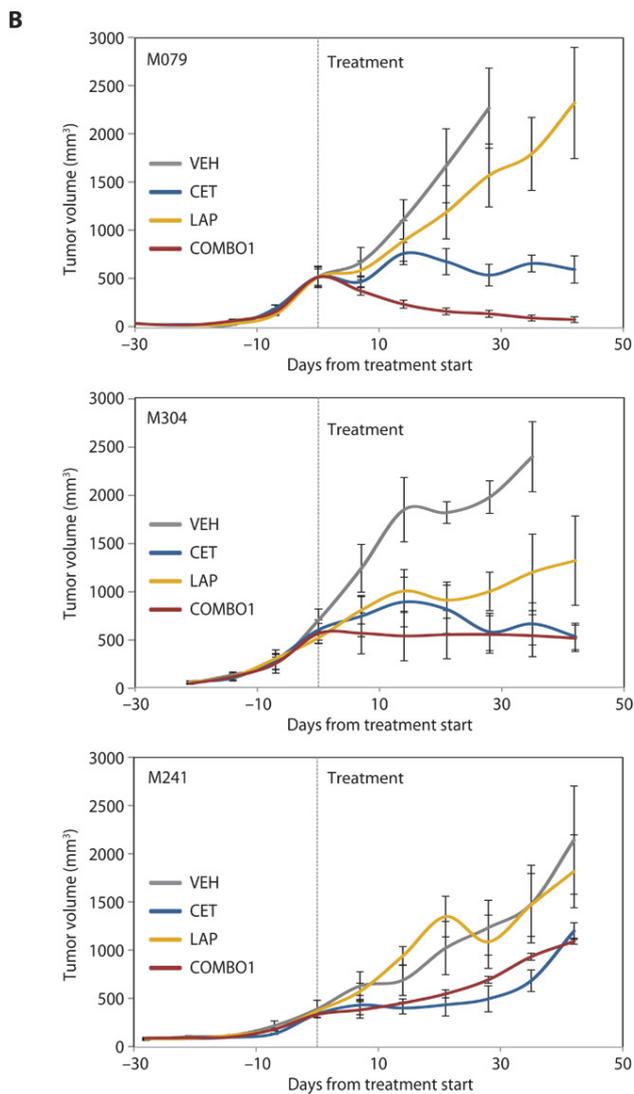


Fig. 2. Effect of cetuximab plus lapatinib: Tumor growth changes.

(A) Waterfall plot of response to cetuximab plus lapatinib after 4 weeks of treatment, compared with tumor volume at baseline, in a population of 21 cases that responded to cetuximab monotherapy with disease stabilization based on the results shown in Fig. 1A. Red bars indicate cases that responded to the combination therapy with massive regressions (mass reduction of more than 75%). Asterisks denote the samples for which growth curves are shown in (B). (B) Representative tumor growth curves in cohorts derived from individual xenopatient, treated with the indicated modalities. $n = 6$ for each treatment arm. Error bars indicate SEM. M079, $P = 0.002$ [combo1 versus cetuximab, two-way analysis of variance (ANOVA)]; M304, $P = 0.575$; M241, $P = 0.749$. VEH, vehicle; CET, cetuximab; LAP, lapatinib; COMBO1, cetuximab plus lapatinib.



Anti-Ki-67 staining of end-of-treatment tumor sections from a representative responder (M079) revealed proliferation defects consistent with the tumor growth curves: cetuximab, but not lapatinib, decreased the number of proliferating cells, which was further abated by combo1 (Fig. 3A). Similarly, cetuximab considerably reduced the phosphorylation of extracellular signal-regulated kinase (ERK) and S6, two canonical EGFR downstream signals along the RAS and phosphatidylinositol 3-kinase (PI3K) pathways, respectively (Fig. 3A). S6 phosphorylation was incrementally depressed by combo1 relative to cetuximab alone, whereas the plateau of ERK deactivation achieved

by cetuximab monotherapy was not further affected by combinatorial treatment (Fig. 3A). Reductions in proliferation index and transducers' activity were accompanied by a corresponding decrement in tumor cellularity at morphological inspection (fig. S5A). Apoptosis, as assessed by active caspase-3 immunoreactivity, was barely detectable in control tumors, was only modestly triggered by combo1 after acute treatment (24 hours), and was again almost imperceptible after prolonged therapy (6 weeks) (fig. S5B). These results suggest that the contribution of combo1 was to shift the steady-state proliferation kinetics of the tumor mass toward a new equilibrium in which attrition was favored over growth, without appreciable induction of cancer cell death.

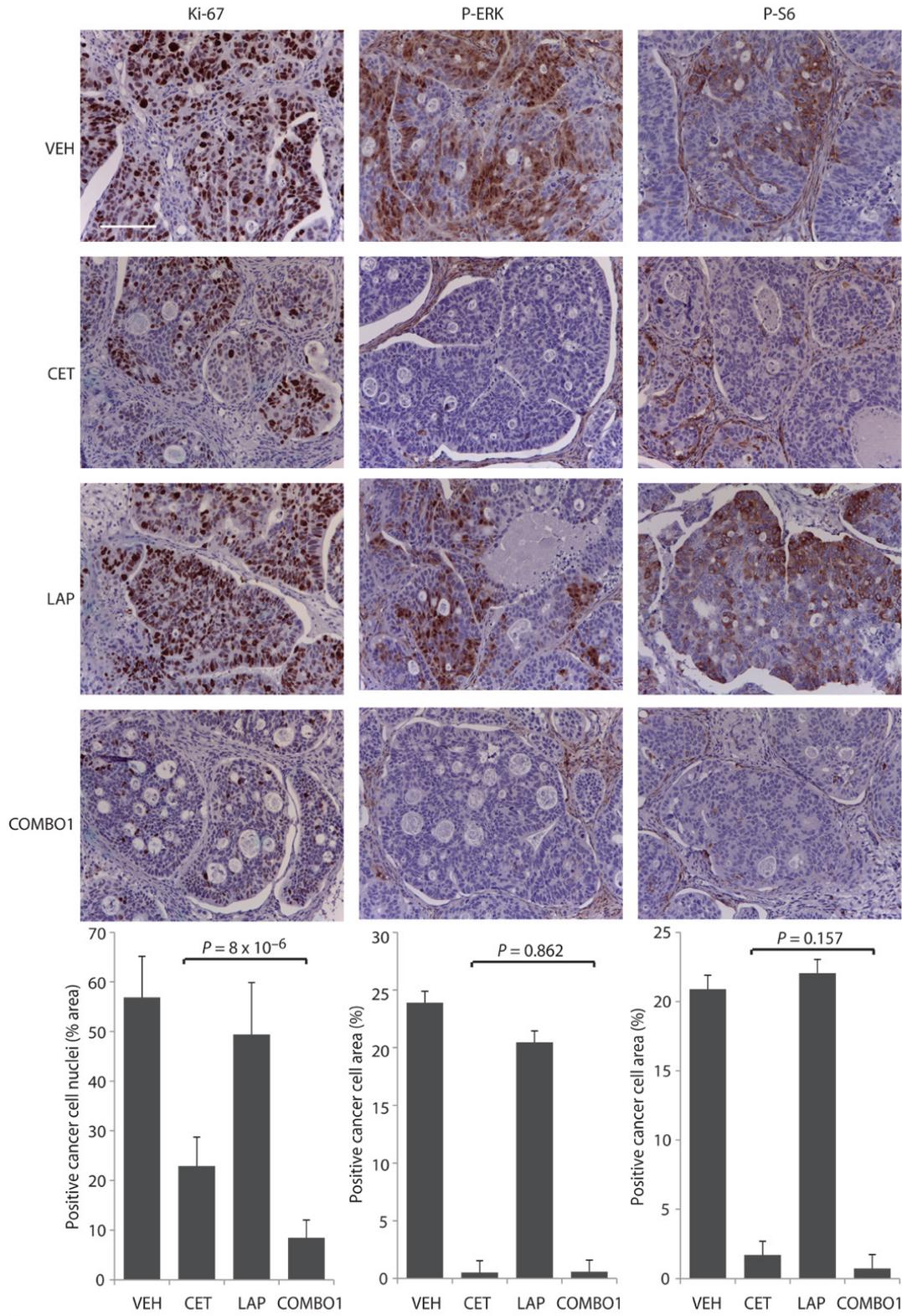
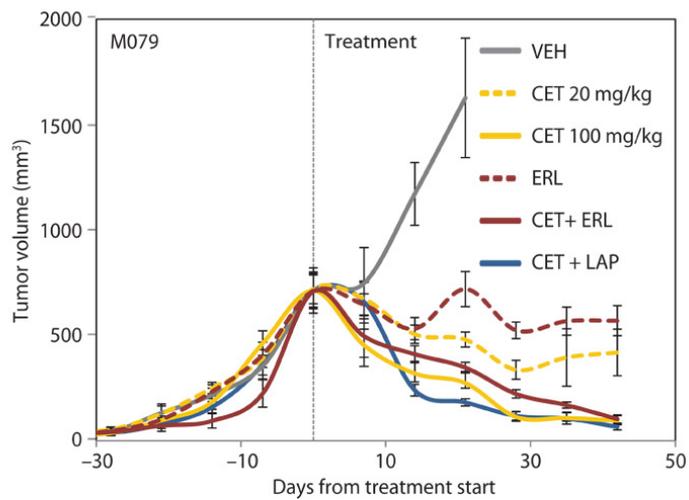
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Fig. 3. Functional effects of cetuximab plus lapatinib or other modalities of EGFR inhibition.

(A) Immunohistochemistry assessment with the indicated antibodies and morphometric quantitation of representative tumors from case M079 at the end of treatment. Results are means \pm standard deviations of 10 fields (40 \times) for each experimental point. Scale bar, 300 μ m. (B) Tumor growth curves of case M079 treated with the indicated modalities. $n = 6$ for each treatment arm. Error bars indicate SEM. $P = 0.009$ [cetuximab plus lapatinib versus cetuximab (20 mg/kg), two-way ANOVA]; $P = 0.007$ [cetuximab plus erlotinib versus cetuximab (20 mg/kg), two-way ANOVA]; $P = 0.017$ [cetuximab (100 mg/kg) versus cetuximab (20 mg/kg), two-way ANOVA]. P-ERK, phospho-ERK; P-S6, phospho-S6; ERL, erlotinib.

Lapatinib inactivates both EGFR and HER2. Therefore, it could synergize with anti-EGFR antibody therapy either by disrupting the enzymatic potential of the EGFR/HER2 complex or by complementing cetuximab for more drastic and specific inhibition of EGFR. To address this issue, we treated M079 with erlotinib, an EGFR-specific inhibitor free from off-target effects at standard concentrations. Similar to lapatinib, erlotinib monotherapy was unable to induce regression, and the combo of cetuximab and erlotinib was as effective as the lapatinib combo in prompting tumor involution (Fig. 3B). As an alternative proof of concept, we boosted EGFR inactivation by increasing the dosage of cetuximab up to fivefold (from 20 to 100 mg/kg). Again, intensified (and selective) EGFR neutralization resulted in tumor shrinkage (Fig. 3B), further pointing to more pronounced blockade of EGFR as a means to achieve better responses.

Expression analysis of parameters that influence response to combination therapies against the EGFR pathway

Concomitant blockade of EGFR by cetuximab and lapatinib/erlotinib was sufficient to turn cetuximab-induced SDs into major ORs in a subset of cases (5 of 21, 23.8%); however, the majority of tumors did not benefit from the addition of small-molecule EGFR inhibitors (Fig. 2A). To explore whether sensitivity to combo1 was more pronounced in SD cases with particularly high EGFR pathway activity, we compared the expression of EGFR family receptors and ligands in combo1 responders versus resistant cases. Although individual differences were quantitatively modest, a general trend toward higher expression of several family members was noticed in responders (HER3, +34%; EGF, +24%; epiregulin, +16%; EGFR, +10%) (fig. S6 and table S1). This suggests that relatively weak increases in the expression of multiple EGFR pathway components might sustain a stronger dependency on EGFR signals and increase the cells' responsiveness to potentiated EGFR inhibition.

We also reasoned that the engagement of survival signals redundant to those transduced by the EGFR pathway and mediated by other cytoprotective cues could be responsible for additional mechanisms of resistance to enhanced EGFR inhibition (23). A recent genome-scale survey of CRC tumors reported considerably higher *IGF2* gene expression (as much as 100-fold increase) in 15% of samples (24). The encoded product, insulin-like growth factor 2 (IGF2), triggers stereotyped antiapoptotic signals that are conveyed by the high-affinity IGF1 tyrosine kinase receptor (IGF1R) and the low-affinity insulin receptor (IR) (25). We detected strong IGF2 overexpression by RT-qPCR in 6 of the 21 cases (28.6%) that had been treated with combo1 (Fig. 4A and table S1). In accordance with the assumption that high IGF2 levels may protect from EGFR pathway inactivation, these 6 IGF2 overexpressors were among the 16 cases in which the cetuximab plus lapatinib combination was not superior to cetuximab alone (6 of 16, 37.5%) (Fig. 4A and table S1). Consistent with ligand-dependent receptor activation, IGF1R tyrosine phosphorylation was strong in two representative models with IGF2 overexpression (M040 and M044) and almost undetectable in two models with lower IGF2 expression (M280 and M328) (fig. S7 and table S1).

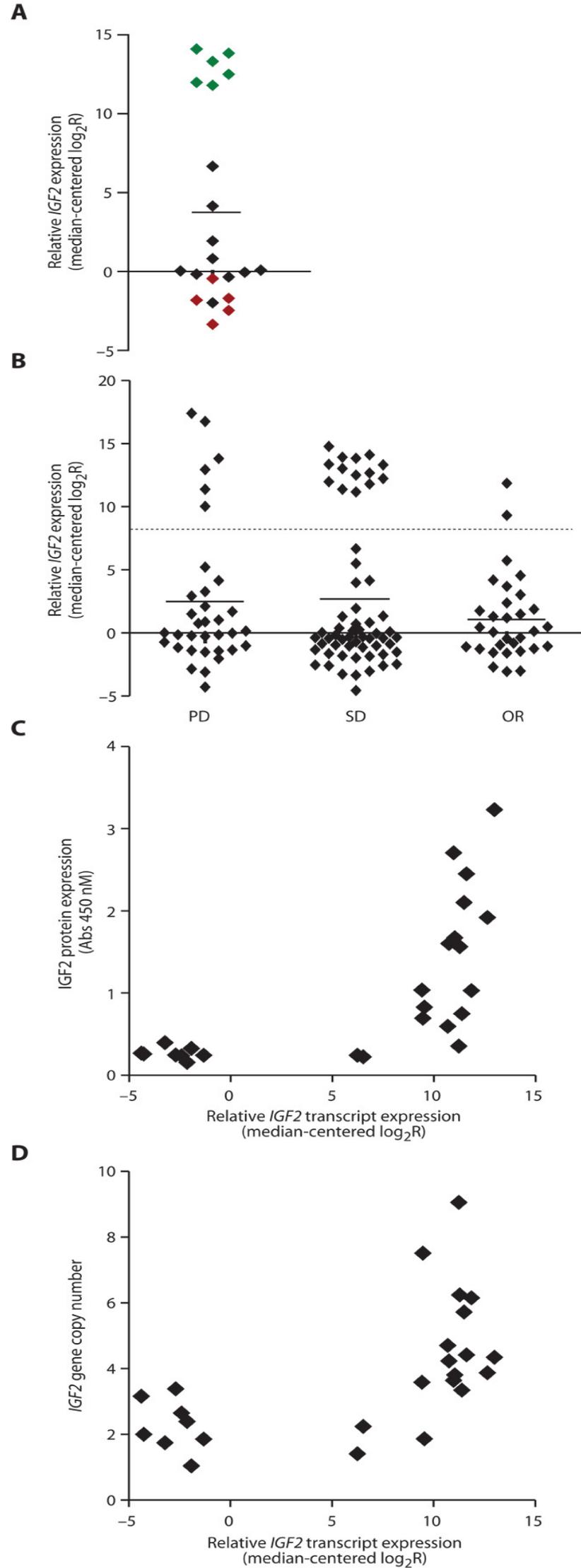


Fig. 4. IGF2 overexpression and reduced sensitivity to cetuximab in xenopatiens.

(A) Gene expression levels of *IGF2* in the 21 cases that were treated with cetuximab plus lapatinib (see Fig. 2A). Green diamonds indicate strong IGF2 overexpressors. Red diamonds indicate cases that responded to cetuximab plus lapatinib with massive tumor shrinkage (>75%). (B) Gene expression of *IGF2* in the same collection of 125 xenopatiens shown in Fig. 1A. Transcript expression was clustered according to cetuximab response. The dotted line indicates the cutoff value for definition of IGF2 outliers (5 standard deviations from the mean of the *IGF2*^{low} subpopulation). See fig. S7 for statistical analysis of frequency distribution of IGF2 expression in xenopatiens. In (A) and (B), continuous lines represent mean transcript expression within each response category. (C) Correlation between *IGF2* transcript expression (*x* axis, RT-qPCR) and protein expression (*y* axis, ELISA) in a subset of 25 cases randomly selected from all samples. Abs, absorbance. (D) Correlation between *IGF2* transcript expression (*x* axis, RT-qPCR) and gene copy number (*y* axis, genomic DNA qPCR) in the same 25 cases shown in (C).

When we reanalyzed the levels of EGFR family ligands and receptors in cases without IGF2 overexpression, the higher expression of EGFR and epiregulin in sensitive cases became more evident (EGFR, +27%; epiregulin, +30%; HER3, +23%; EGF, +19%) (fig. S8 and table S1). This further credentials high EGFR pathway activity as a determinant of responsiveness to potentiated EGFR blockade in cases without other obvious mechanisms of desensitization.

Overexpression of IGF2 and reduced sensitivity to cetuximab: Correlations in xenopatiens

Extension of IGF2 expression analysis to the entire xenopatient collection (table S1) confirmed the presence of a defined subpopulation of IGF2 overexpression outliers (fig. S9). Although the association was not statistically significant by Fisher's exact test ($P = 0.078$), a 3.6-fold enrichment of IGF2 outliers was detected in SD cases (14 of 60, 23.2%) compared to cases that responded with tumor shrinkage (OR) (2 of 31, 6.4%) (Fig. 4B). The percentage of tumors with increased IGF2 expression was higher in SDs than in overtly resistant tumors (PD) (6 of 34, 17.6%) (Fig. 4B). These gene expression data were obtained from passaged samples, indicating autocrine production of IGF2 by cancer cells, similar to that described for EGFR family ligands and receptors. Changes in IGF2 expression, consistent with RT-qPCR data, were confirmed at the protein level by enzyme-linked immunosorbent assay (ELISA) in a subset of 25 cases randomly selected from all samples (Fig. 4C). qPCR gene copy number analysis, performed on the same 25 tumors subjected to ELISA assays, revealed that a subset of samples with higher levels of IGF2 also displayed copy number gains of the *IGF2* locus (Fig. 4D), in accordance with previous findings (24).

IGF2 is part of a complex network that encompasses the canonical receptor IGF1R, a second growth factor with stimulating activity (IGF1) and additional components with inhibitory functions, including six IGF binding proteins (IGFBPs) that limit ligand bioavailability, as well as one receptor (IGF2R) that targets the ligands for degradation in the absence of signal transduction (25). Expression analysis of all these genes did not reveal substantial differences between IGF2 overexpressors and the rest of the cases (fig. S10 and table S1). Similarly, IGF2 levels were not consistently affected by cetuximab in the 14 sensitive models for which expression data after 72 hours and 6 weeks of antibody treatment were available (fig. S11 and table S2). Hence, IGF2 activity in IGF2 outliers depended on intrinsic expression rather than on modulation by regulatory factors. Moreover, IGF2 expression was not influenced by EGFR blockade.

Overexpression of IGF2 and reduced sensitivity to cetuximab: Correlations in patients

To assess the clinical relevance of these results, we explored whether IGF2 overexpressors were enriched in tumors from patients who exhibited attenuated response to anti-EGFR antibodies. We started by mining gene expression profiles from a publicly available microarray data set comprising 48 *KRAS* wild-type CRC liver metastases from patients treated with cetuximab monotherapy (GSE5851, table S1) (20). In agreement with results in xenopatiens, a distinct subpopulation featuring strong IGF2 overexpression (more than fourfold above the median expression in the entire data

set) was detected in many cases categorized as SDs (10 of 17, 58.8%) but not in ORs (0 of 6, $P = 0.051$ by Fisher's exact test) (Fig. 5A); IGF2 overexpressors were less represented among PDs (5 of 25, 20%) (Fig. 5A).

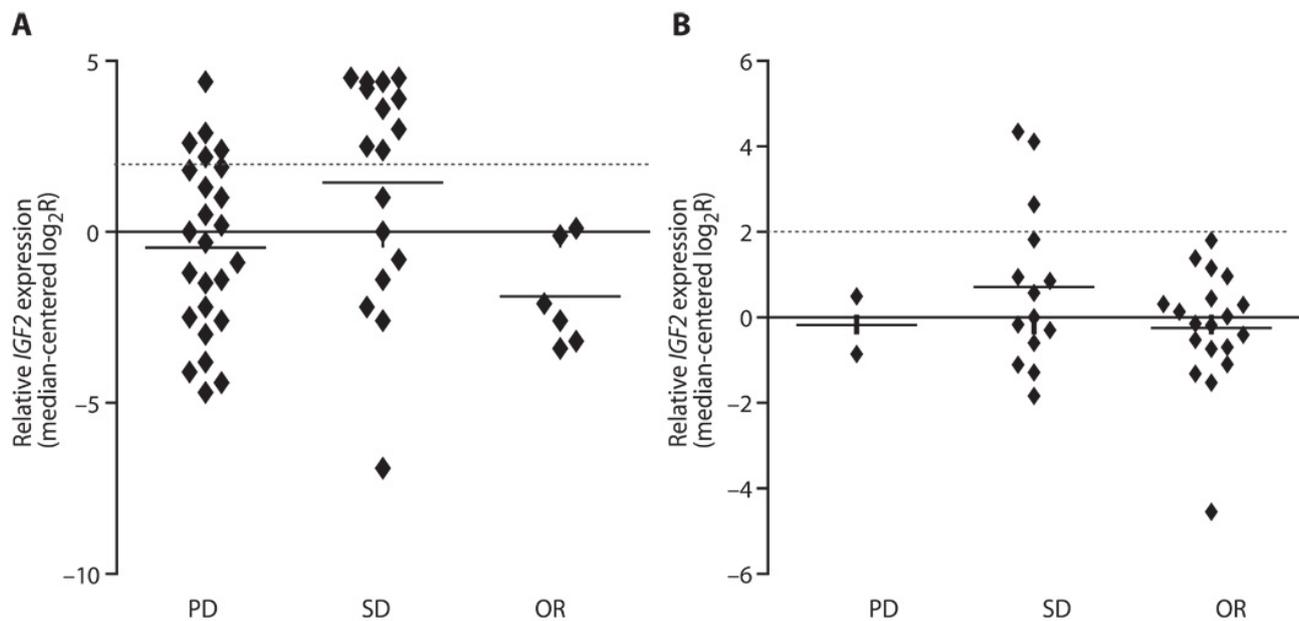


Fig. 5. IGF2 overexpression and reduced sensitivity to cetuximab in patients.

(A) Gene expression of *IGF2* in 48 patients with *KRAS* wild-type metastatic CRCs treated with cetuximab monotherapy. (B) Gene expression of *IGF2* in 35 patients with quadruple negative primary CRCs treated with cetuximab and irinotecan in the chemorefractory setting. Transcript expression was clustered according to cetuximab response. Dotted lines indicate the cutoff value (more than fourfold above the median expression in the entire data set) for definition of strong IGF2 overexpressors. Continuous lines represent mean transcript expression within each response category.

Then, we analyzed IGF2 expression in a retrospective cohort of 35 quadruple negative cases from primary CRCs collected at our institutions and annotated for therapeutic responses to cetuximab and irinotecan in the chemorefractory setting (table S1) (26). Again, samples featuring more than fourfold IGF2 overexpression segregated in the SD category (3 of 14, 21.4%), with no IGF2-high cases among ORs (0 of 19, $P = 0.067$ by Fisher's exact test) or PDs (0 of 2) (Fig. 5B). Also in patient material, in agreement with results in xenopatients, IGF1R was highly phosphorylated in one representative IGF2-overexpressing tumor (S0307F111a) and weakly phosphorylated in another case with lower IGF2 expression (S0307F118a) (fig. S12 and table S1).

In aggregate, when combining both data sets, more than fourfold overexpression of IGF2 was observed in 41.9% of patients whose tumors stabilized on anti-EGFR antibodies (13 of 31) and in none of the patients who responded with tumor regression (0 of 25) ($P = 0.0002$ by Fisher's exact test) (Fig. 5, A and B, and table S1). Among the 27 patients with fully resistant tumors, IGF2 overexpression was observed in 5 cases (18.5%) (Fig. 5, A and B, and table S1). When coupled with results in xenopatients, these findings strongly support the role of IGF2 as a determinant of reduced sensitivity to EGFR-targeted therapies in mCRC.

Overexpression of IGF2 and reduced sensitivity to cetuximab in cell cultures

We undertook functional studies in cell cultures to validate the possible role of IGF2 in blunting sensitivity to anti-EGFR antibodies. On the basis of gene expression data obtained by Illumina beadchip arrays in a panel of 151 CRC cell lines (table S4), we selected two models that featured different endogenous levels of IGF2 (NCI-H508, low expression; HDC142, high expression). Differences in gene expression were confirmed at the transcript level by RT-qPCR (Fig. 6A) and at the protein level by ELISA (Fig. 6B). Mutational profiling showed that both cell lines are wild type for *KRAS* (exons

2 to 4), *NRAS* (exons 2 and 3), *HRAS* (exons 2 and 3), and *BRAF* (exon 15) (27, 28). HDC142 is wild type for *PIK3CA* (exons 9 and 20), whereas NCI-H508 carries a *PIK3CA* exon 9 mutation (E545K) (27, 28), which has been demonstrated not to influence response to anti-EGFR antibodies (7). Hence, the genetic makeup of these cell lines is congruent with the clinical observations about the mutational status of CRC patients most likely to respond to anti-EGFR antibodies.

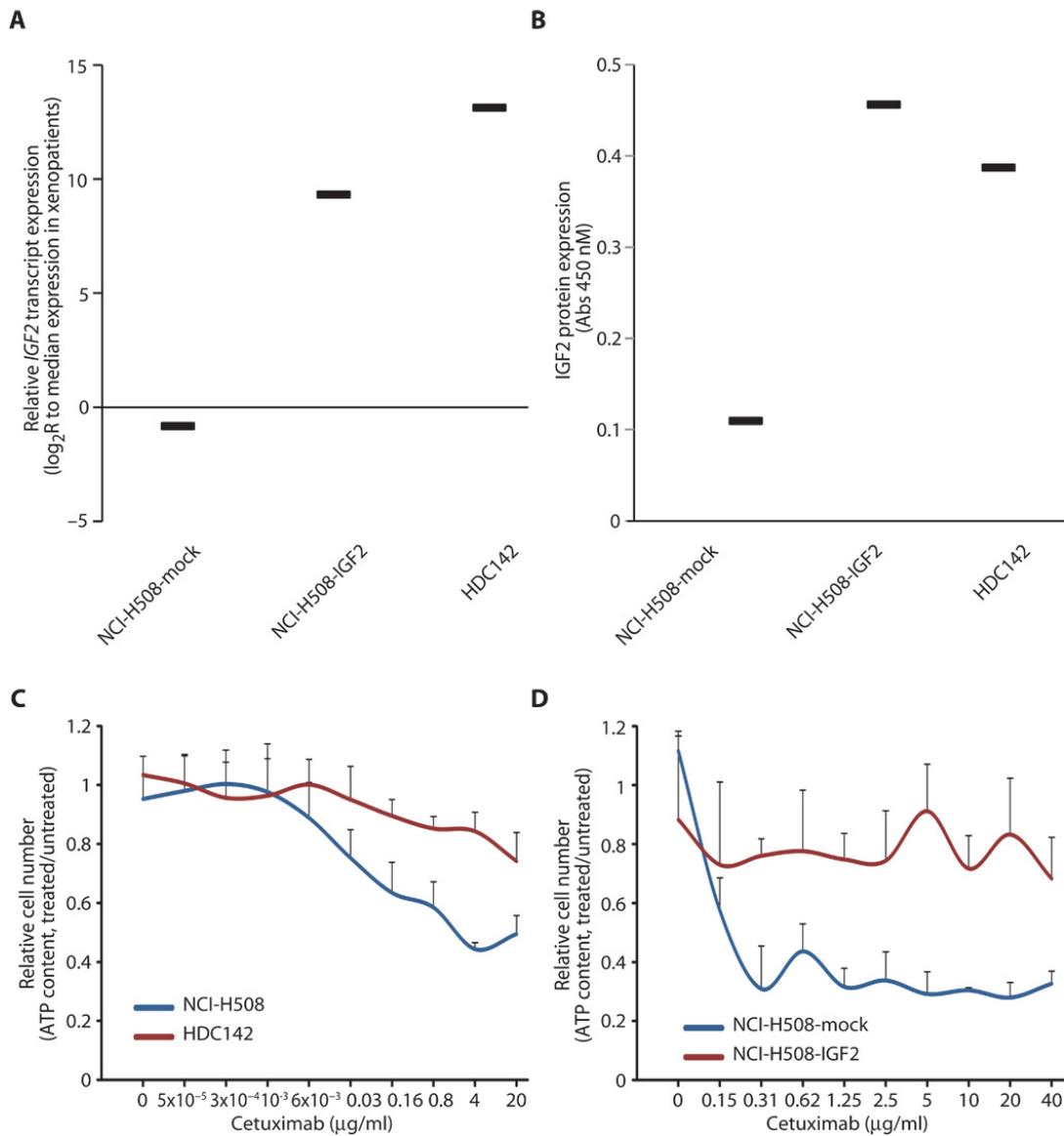


Fig. 6. IGF2 overexpression and reduced sensitivity to cetuximab in cell lines.

(A) *IGF2* transcript expression (RT-qPCR) in the indicated cell lines, relative to median *IGF2* expression in xenopatients. Results are the mean of one experiment performed in technical triplicate. (B) *IGF2* protein expression (ELISA) in the culture supernatant of the indicated cell lines. Results are the mean of one experiment performed in technical triplicate. (C) Sensitivity of NCI-H508 and HDC142 to increasing doses of cetuximab. Results are means \pm standard deviation of two independent experiments performed in quadruplicate. $P = 1 \times 10^{-25}$ (two-way ANOVA). (D) Sensitivity of control NCI-H508 (NCI-H508-mock) and NCI-H508 ectopically overexpressing IGF2 (NCI-H508-IGF2) to increasing doses of cetuximab. Results are means \pm standard deviation of two independent experiments performed in quadruplicate. $P = 8 \times 10^{-12}$ (two-way ANOVA).

In line with *in vivo* results, IGF2 content dictated sensitivity to EGFR blockade, with modest growth inhibition in IGF2-overexpressing HDC142 cells (at cetuximab concentrations as high as 20 µg/ml) and more pronounced inhibition in low-IGF2 NCI-H508 (Fig. 6C). Similarly, exogenous transduction of NCI-H508 with IGF2-encoding lentiviral vectors (Fig. 6, A and B) resulted in weakened response to EGFR inactivation compared with controls: even with higher concentrations of cetuximab (up to 40 µg/ml), the inhibition plateau was about 20% in IGF2 ectopic overexpressors versus 70% in mock-transduced cells (Fig. 6D). Collectively, these *in vitro* results solidify the notion that elevated IGF2 expression decreases CRC responsiveness to anti-EGFR antibodies.

Targeting the IGF2 pathway to improve response to cetuximab

We reasoned that pharmacologic interception of IGF2-driven survival signals may sensitize tumors to EGFR inhibition. Many drugs targeting the IGF1/IGF2 pathway are in late-stage clinical studies, in particular monoclonal antibodies and small-molecule inhibitors of IGF1R (25). Among them, the adenosine triphosphate (ATP)-mimetic IGF1R/IR inhibitor BMS-754807 (29, 30) is one of the most advanced compounds, although the enrolment into trials for this drug has been recently halted because of a lack of efficacy in unselected patients (31). Prompted by the assumption that high-level overexpression of IGF2 could be exploited as a predictive biomarker for molecular stratification of responders, we investigated whether concomitant inactivation of EGFR (by cetuximab) and IGF1R/IR (by BMS-754807) could be beneficial in IGF2-overexpressing CRC tumors.

We tested the efficacy of BMS-754807 in four IGF2-overexpressing xenopatient models (M040, M044, M105, and M380) and one control with low IGF2 levels (M053). For each case, the original tumor specimen was passaged *in vivo* until we had enough mice for concomitant evaluation of four treatment arms: (i) vehicle; (ii) cetuximab alone; (iii) BMS-754807 alone; (iv) cetuximab and BMS-754807 (combo2). BMS-754807 alone slightly retarded tumor growth, regardless of IGF2 expression (Fig. 7A and fig. S13A). Cetuximab monotherapy was confirmed to induce disease stabilization with the exception of case M105, which had experienced SD in our initial screening but proved to be refractory to EGFR inhibition in this new set of experiments (Fig. 7A and fig. S13A). The antitumor activity of combo2 was superior to that of either monotherapy in the four IGF2 overexpressors, with variable induction of tumor shrinkage in M040, M044, and M380, and delay of tumor growth in M105 (Fig. 7A and fig. S13A). Conversely, M053 xenopatient models, which had normal IGF2 levels, did not receive any benefit from combo2 compared with cetuximab alone (Fig. 7A). This indicates that BMS-754807 is effective only in the presence of high IGF2 content, supporting compound specificity.

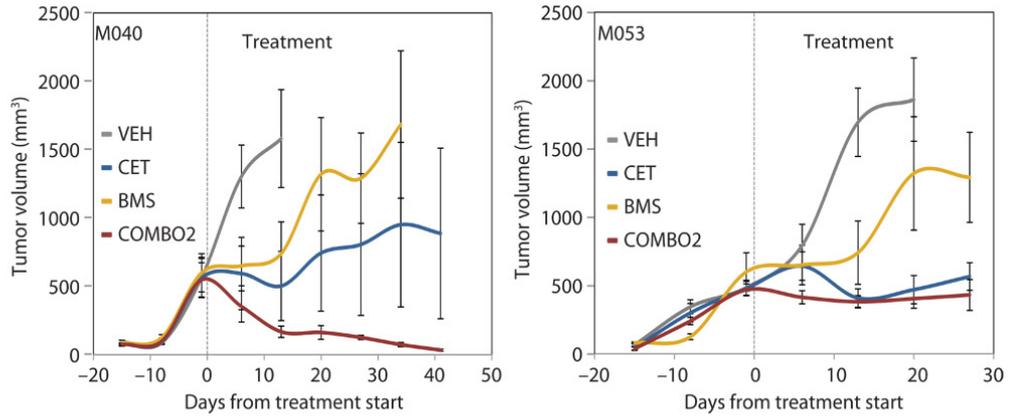
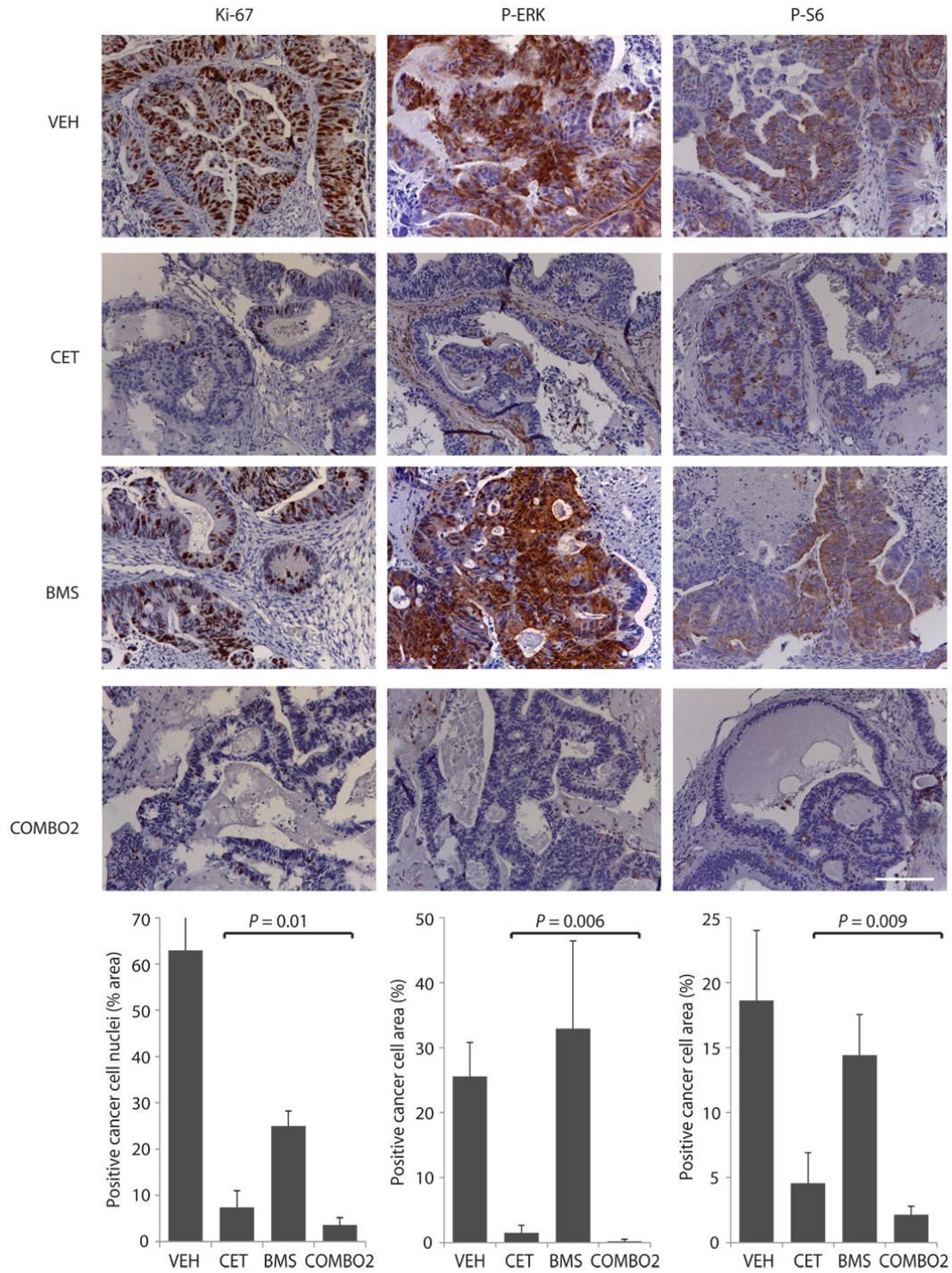
A**B**

Fig. 7. Effect of IGF1R targeting in IGF2-overexpressing xenopatiens.

(A) Tumor growth curves of case M040 (high IGF2 expression) and case M053 (low IGF2 expression) treated with the indicated modalities. $n = 6$ for each treatment arm. Error bars indicate SEM. M040, $P = 0.246$ (combo2 versus cetuximab, two-way ANOVA); M053, $P = 0.754$. (B) Immunohistochemistry assessment with the indicated antibodies and morphometric quantitation of representative tumors from case M040 at the end of treatment. Results are means \pm standard deviation of 10 fields (40 \times) for each experimental point. Scale bar, 300 μm . BMS, BMS-754807; COMBO2, cetuximab plus BMS-754807.

Anti-Ki-67 staining of a representative IGF2-overexpressing tumor (M040) indicated that single-agent cetuximab or BMS-754807 was sufficient to decrease cell proliferation, which was further diminished by combination therapy (Fig. 7B). Again, similar to combo1, no evident effect on apoptosis was detected (fig. S13B). At the signaling level, the combination of cetuximab and BMS-754807 was more potent than cetuximab alone in lowering the phosphorylation levels of S6 and ERK (Fig. 7B). Histologically, the posttreatment tissue of tumors responsive to combo2 displayed reduced cellularity and pseudoglandular differentiation, with acinar structures lined by pluristratified epithelium and embedded in large necrotic areas (fig. S13B). Together, these results provide strong indication that dual blockade of EGFR and IGF2 signaling might be beneficial to improve response in cases that display incomplete sensitivity to anti-EGFR antibodies and aberrantly high IGF2 expression. The observation that susceptibility to treatment was dictated by the degree of IGF2 expression suggests that evaluation of IGF2 content could be exploited to select for potentially responsive patients.

DISCUSSION

Disease stabilization rather than marked regression is the commonest consequence of anti-EGFR therapy in “responsive” mCRC patients (3, 4). It is therefore crucial to identify biologic vulnerabilities that, when targeted (or cotargeted) pharmacologically, can optimize the extent of tumor volume reduction. By generating hypotheses and validating response predictors in a platform of mCRC xenopatiens, we discovered that the limited responsiveness of many CRC tumors to anti-EGFR antibodies can be explained, at least in some cases, by two independent mechanisms: inadequate EGFR inactivation and complementation of EGFR blockade by an IGF2-dependent rescue pathway.

A gene expression survey of EGFR pathway components in xenopatiens, coupled with annotation of response to cetuximab, revealed that the relative levels of EGFR, HER3, and a number of EGFR ligands were elevated not only in cases that had undergone regression—as expected in tumors that were reliant on EGFR signals—but also in partially sensitive cases that had experienced disease stabilization as best response. This analysis confirms previous clinical observations showing a correlation between *EGFR* gene copy number gain or increased expression of amphiregulin/epiregulin and response to cetuximab (16–21), and extends expression data to a wider spectrum of EGFR family receptors and ligands. The finding that mCRC cases with intermediate sensitivity to EGFR inhibition still had high expression of EGFR pathway components lends molecular rationale to treatment modalities aimed at more powerful inactivation of EGFR signals. Consistently, we observed superior activity of combination therapies with cetuximab and EGFR small-molecule inhibitors (lapatinib or erlotinib) in a fraction of SD cases that specifically exhibited increased levels of EGFR, HER3, EGF, and epiregulin. The notion that more effective shrinkage was the consequence of more complete EGFR inactivation was further supported by the use of higher concentrations of cetuximab alone, which mimicked the outcome of the combination therapies.

Preclinical experimentation has provided evidence that combined blockade of EGFR family receptors is more effective than single-agent treatment in inducing shrinkage of tumors with EGFR dependencies (32). For example, in transgenic mice bearing lung tumors with drug-sensitizing EGFR mutations, dual targeting with cetuximab and the irreversible EGFR/HER2 small-molecule inhibitor afatinib increased the frequency of complete responses compared with either monotherapy (33). Similarly, we have recently demonstrated that only a combination of cetuximab (or pertuzumab, a monoclonal antibody that uncouples EGFR/HER2 heterodimers) and lapatinib leads to massive regression of *HER2*-amplified mCRC tumors in xenopatiens (10). In the clinic, combined inhibition of HER2 by lapatinib and the anti-HER2

antibody trastuzumab resulted in higher rates of pathological complete response (in the early neoadjuvant setting) and longer PFS and OS (in the metastatic setting) in patients with HER2-positive breast cancer (34, 35). A recent study has demonstrated encouraging activity of combined treatment with cetuximab and erlotinib in 37 chemorefractory patients with *KRAS* wild-type mCRC tumors (36): the response rate (41%) was definitely higher than that achieved by single-agent anti-EGFR antibodies, which is normally about 15 to 17% (3, 4). Our results agree with these clinical findings and offer rational direction to efforts aimed at facilitating the adaptive allocation of patients to more appropriate treatment groups when initial therapies appear to have modest effects; in this vein, a strategy that, in our opinion, should deserve attention is adding EGFR small-molecule inhibitors to anti-EGFR antibodies in patients in whom cetuximab or panitumumab induces only transient stabilization. On the basis of our preclinical findings, patients likely to benefit the most from small molecule–antibody combinations would be those whose tumors have higher expression of EGFR family ligands and receptors; however, patient stratification based on continuous variables such as subtle changes of gene expression may prove daunting due to the difficulty in setting unambiguous cutoff criteria.

More potent EGFR inhibition was not sufficient to convert all SDs into regressions, most likely due to the presence of signals that activated rescue pathways. Here, we demonstrate that a portion of xenopatiens (23.2%) that exhibited partial sensitivity to cetuximab featured outlier overexpression of the prosurvival cytokine IGF2, whereas almost no evidence of IGF2 overexpression was found in full responders. If we consider only SDs in which dual EGFR targeting by cetuximab and lapatinib was unproductive, the percentage of IGF2 outliers increased to 37.5%.

Cell line–based approaches have documented the association between low sensitivity to *EGFR* blockade and high IGF signaling in other tumor contexts. In *EGFR*-amplified squamous epidermoid carcinoma, acquired resistance to the EGFR small-molecule inhibitor gefitinib was explained with reduced expression of IGFBP-3 and IGFBP-4 (37), both of which negatively modulate IGF1R by sequestering the IGF ligands. In *EGFR*-mutant lung cancer, increased IGF1R activation was shown to mediate chromatin modifications that confer a reversible state of “drug tolerance” to gefitinib (38). In the setting of colorectal cancer, our results indicate that IGF2 is to be considered a response modifier and not a conventional determinant of resistance: indeed, the major dichotomy in the distribution of IGF2 overexpressors was not between fully resistant and responsive cases, but rather between partially sensitive and totally sensitive cases, respectively enriched for and lacking IGF2 overexpressors. Tumors with high IGF2 levels were present in some xenopatiens and patients that proved to be completely refractory to cetuximab, but their prevalence was lower than that observed in SDs. Whether this lack of response was sustained by IGF2 overproduction or by other unknown mechanisms of resistance remains to be determined.

In line with the observation that increased expression of IGF2 could mitigate sensitivity to EGFR inhibition, we found that the antitumor effects of cetuximab were potentiated by disruption of IGF1R signaling in representative IGF2-overexpressing xenopatiens. This effect was specific because it was not recapitulated in a model with low IGF2 expression. Recent clinical trials in patients with *KRAS* wild-type CRC have shown that combined therapy with anti-EGFR and anti-IGF1R antibodies did not improve response compared with EGFR-targeted monotherapy (39, 40). In these studies, no selection based on IGF2 expression levels was performed. Moreover, because we observed therapeutic cooperation with an IGF1R ATP-competitive compound rather than a monoclonal antibody, we cannot exclude that only the association of an anti-EGFR antibody and an anti-IGF1R small-molecule inhibitor might have clinical activity. According to information available on the Web (<https://clinicaltrials.gov/ct2/results?term=bms-754807+AND+cetuximab&Search=Search>), a phase 1 dose escalation study of BMS-754807 and cetuximab in colorectal cancer has been conducted, but results have not been disclosed and, again, patients did not undergo any kind of upfront molecular stratification. More generally, on the basis of negative results from initial phase 3 trials, there is now considerable discussion about the validity of IGFs and IGF1R as therapeutic targets in cancer. The prevailing view is that the apparent lack of efficacy shown by IGF/IGF1R inhibitors can be largely attributed to the fact that no predictive biomarkers to select for potential responders have been identified (25). We report here a molecular lesion in the IGF pathway that (i) identifies a tumor subset (and a patient subpopulation) with discernible biological and clinical characteristics, (ii) acts as a predictor of response, and (iii) can be targeted successfully by a clinically relevant inhibitor. Although IGF2 overexpression was not always the consequence of high-grade gene amplification, the levels of transcript

up-regulation were sufficiently high that they could be easily extracted as discrete information from gene expression data (similar to the digital output of mutational annotations), thereby alleviating concerns about the definition of reliable cutoff values. This streamlines the portability of our results for clinical application and supports the prospective assessment of IGF2 levels in mCRC for a twofold purpose: to stratify a CRC patient subpopulation with a low chance of major response to anti-EGFR antibodies, and to implement a patient enrichment strategy for identification of cases likely to benefit from co-inhibition of EGFR and IGF2 signals.

One limitation of this study is that the only measurable endpoint in xenopatient is response, whereas more definitive parameters such as PFS and OS cannot be reliably evaluated. Although we believe that our results may contribute to increasing the frequency of massive regressions in CRC patients, we cannot anticipate whether this effect will have an impact in terms of survival benefit. We have recently shown that CRC patients who respond to anti-EGFR antibodies with early tumor shrinkage have longer median time to progression and median OS than patients with delayed and less substantial tumor size changes, both in the chemorefractory setting (41) and in first-line therapy (42). This observation suggests that the combination therapies proposed here will positively influence not only patients' response rates but also long-term outcomes. However, retrospective and prospective clinical studies are needed to rigorously address this issue.

In summary, we tested the efficacy of rational drug combinations with the objective of improving the outcome of EGFR inhibition in mCRC. The observation that dual EGFR blockade led to substantial tumor shrinkage in cases that were only partially sensitive to anti-EGFR monotherapy may provide clinicians with ways to more proficiently tackle the ill-defined management of SD. The finding that IGF2 overexpression correlated with reduced responsiveness to anti-EGFR antibodies and predicted sensitivity to inhibitors of IGF2 signaling refines the list of EGFR biomarkers with the addition of a functionally meaningful and therapeutically actionable response modifier.

MATERIALS AND METHODS

Study design

This study was designed to identify mechanisms that attenuate sensitivity to anti-EGFR antibodies in CRC and find therapies that, when combined with EGFR blockade, may improve response and result in massive tumor regression. In the first part of the study, cohorts of nonobese diabetic/severe combined immunodeficient mice bearing tumors expanded from 125 independent patient-derived xenografts (12 or 24 mice for each original sample) were treated with cetuximab monotherapy to identify cases with partial sensitivity (SD). In the second part, responses were correlated with molecular profiles (gene expression data obtained from oligonucleotide microarrays and/or RT-qPCR) to extract expression features specifically segregating in the SD category. In the third part, molecular associations between gene expression and response to therapy were extended to patients using clinically annotated data sets. Finally, potential targets identified by expression analysis were inhibited with specific compounds, alone and in combination with cetuximab, in patient-derived xenografts. Information on specimen collection, implantation and procedures in mice, molecular and bioinformatics analyses, cell-based experimentation, immunohistochemistry, and histopathological analysis, as well as details regarding sample number and replication in assays, are given in figure legends and Supplementary Materials. All values for quantitation of immunohistochemistry images and tumor growth curves in animal experiments were recorded blindly.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Substitution of human stroma with mouse stroma after xenografting.

Fig. S2. Response to cetuximab and expression of EGFR family receptors and ligands.

Fig. S3. Expression changes of EGFR family receptors and ligands during cetuximab therapy.

Fig. S4. Identification of cases selected for combination therapy with cetuximab plus lapatinib.

Fig. S5. Effect of cetuximab plus lapatinib: cellularity and apoptosis.

Fig. S6. Response to cetuximab plus lapatinib and expression of EGFR family receptors and ligands.

Fig. S7. IGF1R phosphorylation in representative tumors from xenopatiens.

Fig. S8. Response to cetuximab plus lapatinib and expression of EGFR family receptors and ligands in cases with low IGF2 overexpression.

Fig. S9. Frequency distribution of *IGF2* expression in xenopatiens.

Fig. S10. Expression of components of the IGF signaling network and association with IGF2 expression.

Fig. S11. Expression changes of *IGF2* during cetuximab therapy.

Fig. S12. IGF1R phosphorylation in representative tumors from patients.

Fig. S13. Effect of IGF1R targeting in IGF2-overexpressing xenopatiens.

Table S1. Baseline microarray data from patients and xenopatiens.

Table S2. Microarray data after 72 hours and 6 weeks of cetuximab treatment.

Table S3. Comparison between response to cetuximab monotherapy and response to cetuximab plus lapatinib.

Table S4. *IGF2* expression in 151 CRC cell lines.

Table S5. List of primers used for gene expression and gene copy number analyses.

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