PIK3CA Mutation/PTEN Expression Status Predicts Response of Colon Cancer Cells to the Epidermal Growth Factor Receptor Inhibitor Cetuximab

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Abstract

Cetuximab is a monoclonal antibody that targets the human epidermal growth factor receptor (EGFR). Although approved for use in EGFR-overexpressing advanced colorectal cancer, recent studies have shown a lack of association between EGFR overexpression and cetuximab response, requiring the identification of novel biomarkers predictive of response to this agent. To do so, 22 colon cancer cell lines were screened for cetuximab response *in vitro* and sensitive and resistant lines were identified. In sensitive cell lines, cetuximab induced a G_0-G_1 arrest without inducing apoptosis. Notably, cetuximab-sensitive but not cetuximab-resistant cell lines were preferentially responsive to EGF-stimulated growth. Whereas neither EGFR protein/mRNA expression nor gene copy number correlated with cetuximab response, examination of the mutation status of signaling components downstream of EGFR showed that cell lines with activating PIK3CA mutations or loss of PTEN expression (PTEN null) were more resistant to cetuximab than PIK3CA wild type (WT)/PTEN-expressing cell lines (14 ± 5.0% versus 38.5 ± 6.4% growth inhibition, mean ± SE; P = 0.008). Consistently, PIK3CA mutant isogenic HCT116 cells showed increased resistance to cetuximab compared with PIK3CA WT controls. Furthermore, cell lines that were PIK3CA mutant/PTEN null and Ras/BRAF mutant were highly resistant to cetuximab compared with those without dual mutations/PTEN loss (10.8 ± 4.3% versus 38.8 ± 5.9% growth inhibition, respectively; P = 0.002), indicating that constitutive and simultaneous activation of the Ras and PIK3CA pathways confers maximal resistance to this agent. A priori screening of colon tumors for PTEN expression status and PIK3CA and Ras/BRAF mutation status could help stratify patients likely to benefit from this therapy. [Cancer Res 2008;68(6):1953–61]

Introduction

The epidermal growth factor receptor (EGFR) signaling pathway is commonly activated in colorectal cancer and has been explored for several years as a target for cancer therapy (1). EGFR is expressed in 30% to 85% of colorectal cancer patients, and the intensity of its expression has been linked to reduced survival (2, 3). Dysregulation of EGFR signaling has been shown to stimulate cell proliferation, angiogenesis, and metastatic spread and to inhibit apoptosis (1, 4). Activation of this pathway occurs after ligand [EGF, transforming growth factor (TGF), amphiregulin] binding to EGFR, which leads to EGFR phosphorylation and oligodimerization at the plasma membrane. This in turn triggers a chain of downstream signaling events that include activation of the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)–Akt, and signal transducer and activator of transcription pathways (4, 5). Cetuximab is a chimeric IgG1 monoclonal antibody that targets the extracellular domain of EGFR, blocking ligand binding to the receptor (1). Based on a randomized phase II clinical trial, cetuximab was approved in the United States in 2004 for use in combination with irinotecan or as monotherapy in EGFR-positive irinotecan-refractory colorectal cancer (6).

Cetuximab, however, has an objective response rate of only 9% when used as a single agent, along with toxicities of diarrhea, skin rash, and infusion reactions (7). There is therefore a clear need for biomarkers predictive of response to cetuximab to maximize likelihood of response while minimizing toxicities and cost.

Whereas cetuximab was initially approved for treatment of patients with EGFR overexpression, the utility of EGFR as a predictive biomarker for cetuximab response has become increasingly controversial (6, 8, 9). One limitation of these studies is that EGFR expression status was frequently measured in the primary tumor, whereas objective response is measured in metastatic lesions. Recent studies have shown variability in EGFR expression between primary and metastatic tumors from the same patient (10, 11), suggesting the need to determine EGFR expression status at the stage and site at which response is determined. The first objective of this study, therefore, was to directly compare EGFR expression and cetuximab response in the same colon cancer cell lines.

In addition to EGFR expression, a further determinant of cetuximab sensitivity may be the presence or absence of mutations that result in constitutive activation of EGFR-mediated signaling. For example, in lung cancer, patients with activating mutations in the EGFR tyrosine kinase domain (encoded by exons 18–21) show significantly greater response to the small molecule inhibitors of EGFR tyrosine kinase activity, gefitinib and erlotinib (12–14). Whereas mutations in the EGFR kinase domain are extremely rare in colon cancer (15), mutations which constitutively activate key signaling mediators downstream of EGFR, particularly K-Ras/BRAF and PTEN/PIK3CA, are more common (16–19). We hypothesized

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therefore that colon tumors with constitutively activated downstream signaling mediators of the EGFR pathway would be refractory to inhibition of the pathway at the receptor level. Indeed, several recent studies have shown a link between K-Ras mutation status and cetuximab response, with tumors wild type (WT) for K-Ras showing improved response to this agent (20–22). However, other studies failed to do so (23).

In the present study, we observed that separation of cell lines according to PIK3CA mutation/PTEN expression status significantly distinguished cetuximab sensitive and resistant cell lines. Particularly, cell lines with both PIK3CA mutation/PTEN loss and Ras/BRAF mutation were highly resistant to cetuximab. Therefore, a priori screening of colon tumors for PIK3CA mutation/PTEN expression and Ras/BRAF mutation status may help identify patients likely to benefit from this therapy.

Materials and Methods

Determination of sensitivity of colon cancer cell lines to cetuximab and EGF. The sources and maintenance of the colon cancer cell lines used in this study have been previously described (24), with the exception of the GEO cell line which was kindly provided by Dr. Z Fan (M.D. Anderson Cancer Center).

For determination of cetuximab sensitivity, 5,000 to 50,000 cells per well (24) were seeded in 96-well plates and treated with 0, 0.01, 0.1, 1, 5, 10, 20, 50, and 100 μg/mL cetuximab for 72 h. For each cell line, one plate was harvested for determination of $t = 0$ absorbance values. Viable cells were determined 72 h posttreatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by measurement of absorbance at 570 nm. The relative rate of cell growth for each cell line was factored into the analysis by subtracting the absorbance at time 0 from both the control and treatment groups. All the experiments were replicated thrice at a minimum.

For determination of sensitivity to EGF, cells were serum starved for 4 h and then treated with 0, 0.5, or 5 ng/mL EGF for 24 to 72 h. For cetuximab/EGF cotreatment experiments, cells were pretreated with cetuximab for 4 h before EGF addition.

HCT116 K-Ras and PIK3CA isogenic cell lines. HCT116 colon cancer cells harbor both activating K-Ras and PIK3CA mutations. Isogenic HCT116 K-Ras and PIK3CA WT and mutant cells were generously provided by the Sasuzaki and Vogelstein/Velculescu Laboratories, respectively (25, 26).

Cell cycle distribution—fluorescence activated cell sorting analysis. For assessment of the effect of cetuximab and EGF on cell cycle distribution, cells were stained with 50 μg/mL propidium iodide overnight and fluorescence-activated cell sorting (FACS) analysis was performed, as previously described (27).

Determination of cetuximab response *in vivo*. For xenograft experiments, 5×10^6 GEO or LIM2405 cells in 200 μL of PBS/Matrigel (1:1) were injected s.c. into the right flank of SCID mice. Tumors were allowed to form for ~1 wk. Animals were then injected with either PBS or cetuximab (10 mg/kg or ~300 μg per mouse) i.p. biweekly for 2 wk, as previously described (28). Upon sacrifice, tumor volume was calculated from measurements of the smallest (s) and longest (l) diameter based on the following formula: volume = $[(s^2 \times l) \times \pi] / 6$.

Determination of EGFR protein and mRNA expression in colon cancer cell lines. EGFR protein expression was determined by Western blot using an anti-EGFR antibody (Cell Signaling Technology). EGFR mRNA expression was determined by quantitative real-time PCR. EGFR-specific primers were as follows: (F, ATGCTCTACAACCCCACCAC; R, GCCCTTCGCACTTCTTACAC). Results were expressed relative to glyceraldehyde-3-phosphate dehydrogenase (primers: F, TCGGAGTCAACGGATTTGG; R, GAATTTGCCATGGGTGGAAT).

Determination of EGFR gene copy number by fluorescence *in situ* hybridization. EGFR copy number was assessed in colcemid-treated cells by standard cytogenetic methods using 0.075 mol/L KCl and Carnoy's fixative (methanol/acetic acid, 3:1). Metaphase chromosomes were hybridized overnight with the dual color LSI EGFR/CEP7 (Vysis locus–specific identifier DNA) probe and counterstained with 4′,6-diamidino-2-phenylindole. This probe has a specific EGFR Spectrum Orange probe and a CEP7 probe, labeled in spectrum green, which hybridizes to the α satellite DNA located at the centromere of chromosome 7 (7p11.1-q11.1). Images were acquired with an epifluorescence microscope (Olympus BX51) connected to a Sensicam QE CCD cooled camera. Ten metaphases for each cell line were analyzed using the fluorescence *in situ* hybridization (FISH) view software (Spectral Imaging). EGFR gene amplification was defined as more than one copy of EGFR locus per chromosome 7, and EGFR polysomy was defined as more than two EGFR loci per nucleus.

Identification of K-Ras, BRAF, PIK3CA, and PTEN mutations in colon cancer cell lines. The mutation status of K-Ras, BRAF (exon 15), PIK3CA (exons 9 and 20), and PTEN for a subset of the cell lines was obtained from the Wellcome Trust Sanger Institute Cancer Genome Project Web site[4] or from previous publications. For cell lines for which the mutation status of one or more of these genes was unknown, genomic DNA was isolated using the Qiagen DNA extraction kit. Primers used for amplification of exon 2 of K-Ras were F, AGGCCTGCTGAAAATGACTGAATA and R, CTGTATCAAAGAATGGTCCTGCAC. Primers used were F, AACACATTTCAAGCCCCAAA and R, GAAACTGGTTTCAAAATATTCGTT for amplification of exon 15 of BRAF; F, GCTTTTTCTGTAAATCATCTGTG and R, CTGAGATCAGCCAAATTCAGT for exon 9 of PIK3CA; and F, CATTTGCTCCAAACTGACCA and R, TACTCCAAAGCCTCTTGCTC (for codon 1023 mutation) and F, ACATTCGAAAGACCCTAGCC and R, CAATTCCTATGCAATCGGTCT (for codon 1047 mutation) for exon 20 of PIK3CA. PTEN expression status was determined by Western blot using an anti-PTEN antibody (Cell Signaling).

Statistical analyses. Differences between two groups were analyzed using an unpaired Student's t test with $P < 0.05$ considered statistically significant. Differences in cetuximab sensitivity between cell lines WT or mutant for specific genes or gene combinations were determined using an unpaired Student's t test with Bonferroni adjustment. Because we performed five separate analyses, $P < 0.01$ was considered statistically significant. For correlative analyses, a Pearson's correlation coefficient was computed with $P < 0.05$ considered statistically significant.

Results

Determination of sensitivity of colon cancer cell lines to cetuximab. To identify colon cancer cell lines with differential response to cetuximab, a panel of 22 colon cancer cell lines was screened for cetuximab response using the MTT assay. As shown in Fig. 1 and Supplementary Fig. S1, a spectrum of sensitivity to cetuximab was identified. Maximal response was observed in the LIM1215, GEO, and SW403 cell lines, where growth was inhibited >70% at the maximum concentration of cetuximab tested. An intermediate response (~50% growth inhibition) was observed in the Caco-2 and SW948 cell lines, whereas minimal response (<35% growth inhibition) was observed in the remaining cell lines (Fig. 1 and Supplementary Fig. S1).

Differential sensitivity of colon cancer cell lines to cetuximab *in vitro* is also observed *in vivo*. To confirm the differential sensitivity of colon cancer cell lines to cetuximab *in vivo*, the sensitive GEO and resistant LIM2405 cell lines were grown as xenografts in SCID mice and treated with 10 mg/kg cetuximab biweekly for 2 weeks. Consistent with the *in vitro* findings, cetuximab inhibited growth of GEO cells by ~75% (205 ± 36 mm^3 compared with 854 ± 201 mm^3 in cetuximab-treated and control mice, respectively), whereas no growth inhibitory effect was observed in LIM2405 cells (999 ± 32 mm^3 versus 934 ± 66 mm^3 in cetuximab-treated and control mice, respectively; Fig. 1*B*).

[4] http://www.sanger.ac.uk/genetics/CGP/cosmic/

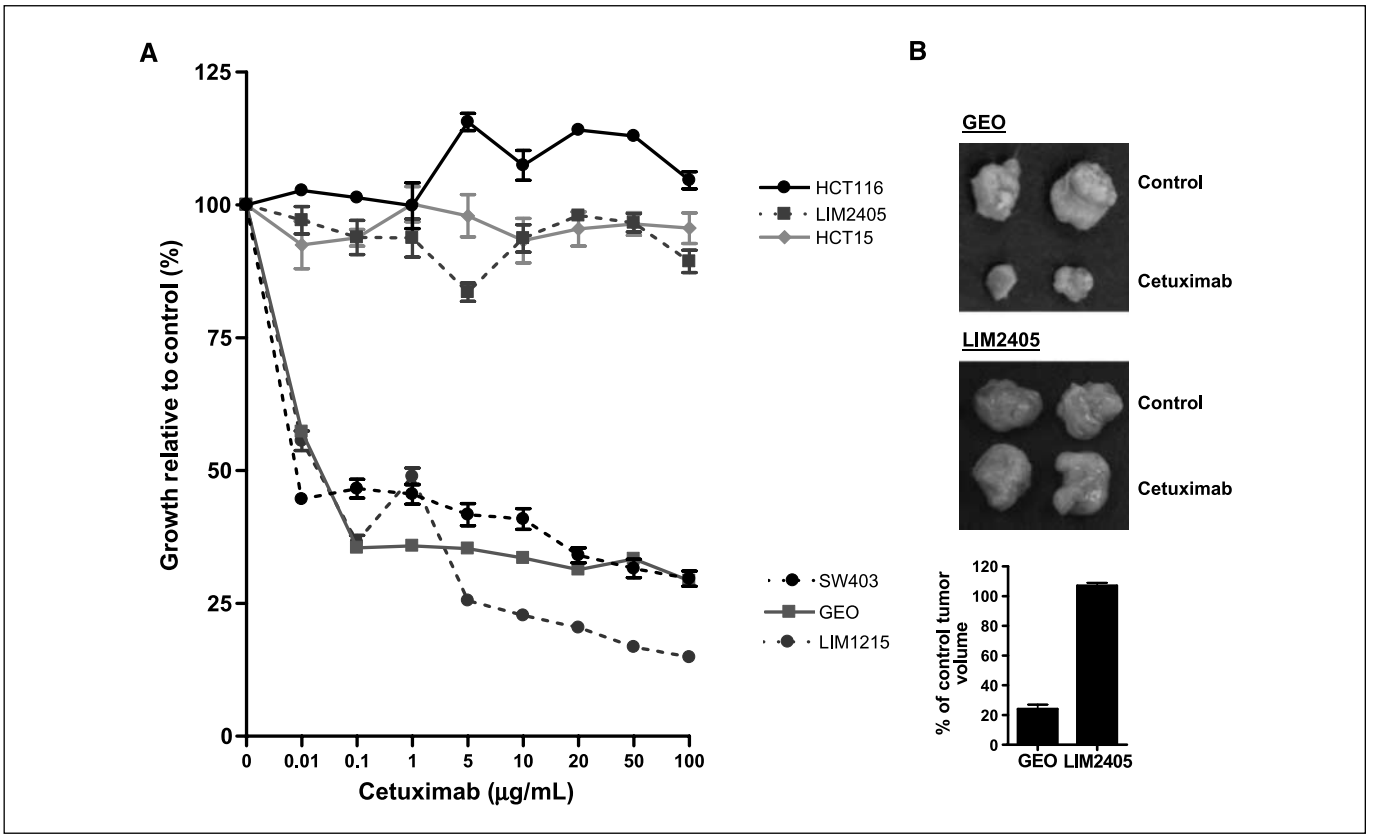

Figure 1. *A,* differential sensitivity of colon cancer cell lines to 72-h cetuximab treatment. Shown for simplicity are the three most sensitive and resistant cell lines of the 22 cell lines screened for cetuximab response. *Points,* mean (n = 3–5 experiments); *bars,* SE. *B,* differential sensitivity of colon cancer cells to cetuximab *in vivo*. 5×10^6 cells of the cetuximab-sensitive (GEO) and cetuximab-resistant (LIM2405) colon cancer cell lines were injected in SCID mice. Once palpable tumors had formed, animals were injected with cetuximab (10 mg/kg) or PBS (control), biweekly, for 2 wk, after which animals were sacrificed, tumors were excised, and tumor volume was calculated, as in Materials and Methods.

Comparable sensitivity profiles of colon cancer cells to cetuximab and erlotinb. To confirm that the sensitivity spectrum of colon cancer cells to cetuximab reflected inhibition of EGFR and its downstream signaling pathway, we assessed the response of 12 of the cell lines to erlotinib (5 μg/mL), a small molecule tyrosine kinase inhibitor that targets the intracellular domain of EGFR. As shown in Supplementary Fig. S2, a significant correlation between response of colon cancer cell lines to cetuximab and erlotinib was observed, consistent with both agents mediating growth inhibition through inhibition of EGFR signaling (r^2 = 0.542, P = 0.006). In contrast, no significant correlation was observed between cetuximab response and response to the mechanistically distinct chemotherapeutic agent, 5-fluorouracil (5-FU; r^2 = 0.019, P = 0.678; Supplementary Fig. S2).

Cetuximab induces a G_0-G_1 arrest in colon cancer cells. To confirm the findings of the MTT assay and to further assess the effect of cetuximab on cell cycle distribution, we determined the effect of cetuximab on cell cycle distribution in the three most sensitive and three most resistant cell lines (Fig. 2*A*–*C*). Consistent with the MTT data, an increase in the percentage of cells in G_0-G_1 and a concomitant decrease in the percentage of cells in S phase were observed in the three sensitive cell lines (LIM1215, GEO, and SW403). No difference in the percentage of cells in G_2-M was observed. In comparison, minimal change in cell cycle distribution was observed in the resistant cell lines (LIM2405, HCT116, and HCT15). Importantly, minimal effects on apoptosis were observed in this cell line panel, either at 24 or 72 hours after cetuximab treatment (Fig. 2*D*), indicating cetuximab elicits a predominantly cytostatic effect in colon cancer cells. In contrast, treatment of SW403 cells with 5 μmol/L 5-FU induced ~50% apoptosis after 72 hours of treatment, demonstrating that these cell lines are not inherently resistant to apoptosis (Fig. 2*D*).

EGFR mRNA, protein expression, or gene copy number do not correlate with cetuximab response in colon cancer cell lines. Because cetuximab targets EGFR, we determined whether basal EGFR mRNA and/or protein expression in the cell line panel correlated with cetuximab response. EGFR mRNA in the 22–cell line panel was assessed by quantitative reverse transcription–PCR (Q-RT-PCR). As shown in Fig. 3*A*, no correlation between basal EGFR mRNA expression and cetuximab response was observed (R^2 = 0.067, P = 0.244). To determine whether basal EGFR protein expression correlated with cetuximab response, EGFR protein expression was assessed in the 22 cell lines. Whereas significant correlation between EGFR mRNA and protein expression was observed (R^2 = 0.35, P = 0.003), no correlation between basal EGFR protein expression and cetuximab response was observed (R = −0.36, R^2 = 0.13, P = 0.099; Fig. 3*B*).

Finally, studies by Moroni et al. suggested that EGFR gene amplification status correlates with response to cetuximab in patients with colon cancer (23). Hence, the EGFR amplification status of the three most sensitive and three most resistant colon cancer cell lines was determined by FISH analysis. As shown in

Fig. 3*C*, no EGFR gene amplification was seen in any of the six colon cancer cell lines tested. Two sensitive (GEO and SW403) and one resistant cell line (LIM2405) contained three copies of EGFR per nucleus; however, in each case, three copies of the chromosome 7 marker CEP7 were also observed indicating this reflected polysomy as opposed to amplification of the EGFR locus. Collectively, therefore, no correlation between cetuximab response and EGFR expression of copy number was observed in the colon cancer cell line panel.

Cetuximab sensitivity correlates with growth response to EGF. Signaling via the EGFR receptor is initiated upon ligand binding (EGF, TGF-α, amphiregulin), with signal transduction primarily through the PTEN/PI3K/AKT and/or Ras/Raf/MAP/MEK/ERK pathways. We speculated, therefore, that cell lines responsive to ligand mediated canonical activation of this pathway for their growth would be most sensitive to cetuximab.

To test this, the proliferative response of the three most cetuximab-sensitive and three most cetuximab-resistant cell lines to exogenous EGF treatment was examined under serum-free conditions (Fig. 4). A significant increase in S phase was observed 24 hours post–EGF treatment (0.5 ng/mL) in the three cetuximab-sensitive cell lines (GEO, LIM1215, and SW403) but not in the three most cetuximab-resistant cell lines (LIM2405, HCT15, and HCT116; Fig. 4*A*). To confirm this result, we also performed MTT assays. Treatment with 0.5 or 5 ng/mL EGF preferentially increased growth of the cetuximab-sensitive cell lines, establishing a clear link between response to the mitogenic effects of EGF and the growth inhibitory effects of cetuximab (Fig. 4*B*). Similar results were observed when response to a 10-fold higher concentration of EGF (5 ng/mL) was tested (Supplementary Fig. S3). Importantly, pretreatment of the EGF-responsive cell lines with cetuximab significantly attenuated the mitogenic effect of EGF (Fig. 4*B*).

Mutation status of PIK3CA and/or PTEN predicts response to cetuximab. Ligand binding to EGFR results in signal transduction via the Ras/Raf/MEK/MAPK and the PI3K/AKT pathway. As mutations that result in constitutive activation of each of these pathways occur at high frequencies in colon cancer, we hypothesized that colon cancer cell lines with constitutively activated signaling downstream of EGFR would not be dependent on ligand binding to EGFR for their growth and, in turn, would be refractory to cetuximab.

Mutation-driven constitutive activation of the PI3K signaling pathway has been reported to occur in ~30% of colon tumors, primarily due to activating mutations in exons 9 and 20 of the PIK3CA gene (18) and, to a lesser extent, due to inactivating PTEN mutations or PTEN promoter methylation (29). The presence of activating PIK3CA mutations in the cell line panel was assessed by literature searches from the COSMIC database and by direct sequencing of exons 9 and 20 of the PIK3CA gene. Mutations in PIK3CA were identified in 8 of the 22 cell lines (Table 1; Supplementary Fig. S5). Separation of cell lines according to PIK3CA mutation status alone did not distinguish cetuximab sensitive from resistant cell lines (16.3 ± 6.0% versus 33.7 ± 6.4% growth inhibition for PIK3CA mutant versus WT cell lines, respectively; $P = 0.08$).

PTEN is a tumor suppressor that acts as a negative regulator of PI3K signaling by converting PIP_3 to PIP_2, and truncating mutations which result in loss of PTEN expression have been reported in ~20% of MSI colon cancers (30, 31). We noted that the KM12 cell line that was PIK3CA WT, yet highly resistant to

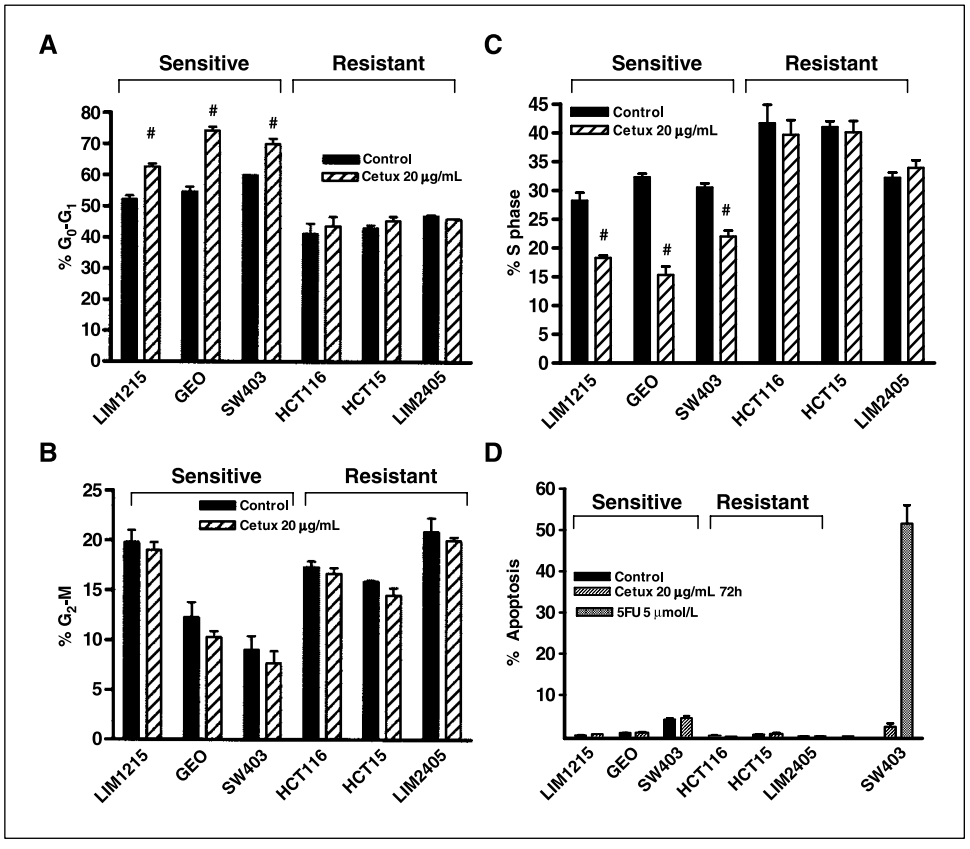

Figure 2. Summary of cell cycle analysis of cetuximab-sensitive (GEO, LIM1215, SW403) and cetuximab-resistant (LIM2405, HCT116, HCT15) colon cancer cell lines. For assessment of cell cycle distribution, cells were treated for 24 h with 20 μg/mL cetuximab (*A–C*). For assessment of apoptosis, cells were treated with 20 μg/mL cetuximab or 5 μmol/L 5-FU for 72 h (*D*). Cell cycle distribution and apoptosis was assessed by propidium iodide staining and FACS analysis. *Points,* mean; *bars,* SE ($n = 3$; #, $P < 0.05$).

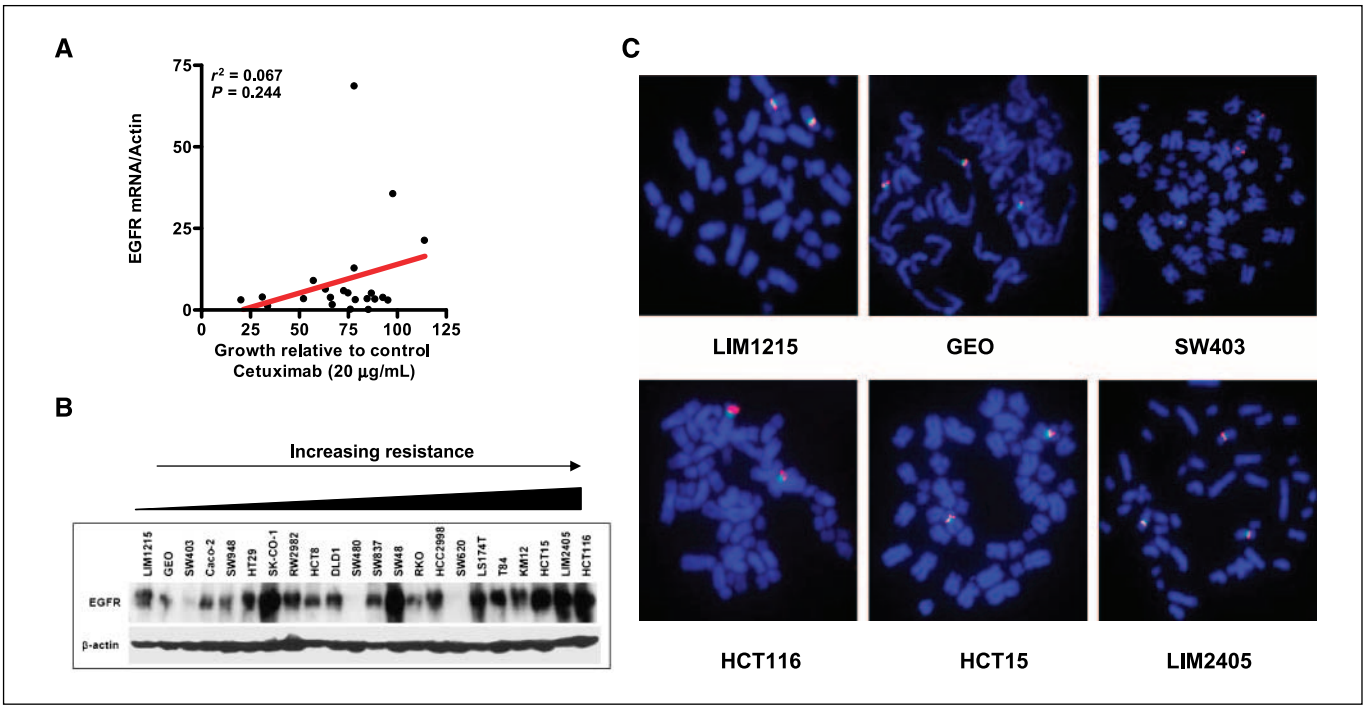

Figure 3. Basal EGFR mRNA, protein expression, or EGFR copy number does not correlate with cetuximab response. *A,* correlation of basal EGFR mRNA expression as assessed in exponentially growing colon cancer cells by Q-RT-PCR and cetuximab response at the 20 μg/mL dose. *B,* EGFR protein expression in the 22 cell lines as assessed by Western blot analysis. *C,* EGFR (*red*) copy number as determined by FISH analysis in the three sensitive and three resistant cell lines. CEP7 (*green*) was used as marker of chromosome 7.

cetuximab, harbors a truncating mutation in PTEN (Wellcome Trust Sanger Institute Cancer Genome Project[5]). The PTEN expression status of the cell line panel was therefore determined by Western blot (Supplementary Fig. S4). In addition to KM12, loss of PTEN expression was also observed in the cetuximab resistant LIM2405 line (Supplementary Fig. S4). Consistent with previous reports, both lines with loss of PTEN expression were derived from MSI colon cancers. Furthermore, the occurrence of PIK3CA mutations and loss of PTEN expression in the cell line was mutually exclusive, as previously reported (32).

Separation of cell lines according to PIK3CA mutation and/or PTEN expression status identified a significant difference in cetuximab sensitivity, with PIK3CA mutant/PTEN null cell lines being significantly more refractory to cetuximab treatment (14.0 ± 5.0% versus 38.5 ± 6.4% growth inhibition for PIK3CA mutant/PTEN null versus PIK3CA WT/PTEN-expressing cell lines, respectively; P = 0.008; Fig. 5*A*).

To further confirm this finding, we examined cetuximab response in a pair of isogenic HCT116 cells provided by the Velculescu/Vogelstein Laboratories, in which either the mutant or WT PIK3CA allele has been deleted by homologous recombination (26). Whereas the PIK3CA mutant HCT116 isogenic cell line was highly resistant to cetuximab, a modest, although statistically significant, response to cetuximab was observed in the PIK3CA WT isogenic line (Fig. 5*B*). Collectively, these findings show that colon cancer cell lines with constitutively active PI3K signaling are refractory to cetuximab.

Mutation status of K-Ras or BRAF does not predict response to cetuximab. Several clinical studies have examined the relationship between Ras mutation status of colon tumors and response to cetuximab. Whereas an initial study did not observe an association (23), more recent studies have shown that colon tumors mutant for K-Ras are less responsive to cetuximab compared with WT tumors (20–22).

To validate and extend these findings, the K-Ras mutation status of the cell line panel was determined by literature searches, searching of the Sanger database or direct sequencing of codons 12 and 13 in exon 2 of the K-Ras gene (Table 1; Supplementary Fig. S5). Of the 22 cell lines in the panel, 13 were mutant and 9 were WT for K-Ras. Separation of the cell lines according to Ras mutation status did not result in a differential response to cetuximab (22.2 ± 6.6% versus 34.7 ± 6.8% growth inhibition for Ras mutant versus WT cell lines, respectively; P = 0.21). The lack of an association between Ras mutation status and resistance to cetuximab was underscored by the observation that the established cetuximab-sensitive cell line GEO (28) and SW403 harbored a mutant K-Ras allele (Fig. 1). To further confirm this finding, we compared cetuximab response in parental HCT116 cells (Ras mutant) and the HCT116 Ras WT isogenic derivative (Hke3) generated by targeted deletion of the mutant K-Ras allele. No difference in cetuximab response, as assessed by cell number 24 hours post–cetuximab treatment, was observed between the Ras mutant and WT clones (Fig. 5*C*).

Mutations in BRAF are frequently observed in colon tumors WT for K-Ras. The BRAF mutation status of the cell line panel was therefore determined by direct sequencing of exon 15. Three cell lines harboring BRAF mutations were identified in the cell line panel (RKO, HT29, and LIM2405). Consistent with the previously

[5] http://www.sanger.ac.uk/genetics/CGP

observed mutual exclusion of Ras and BRAF mutations in colon tumors, the three cell lines with BRAF mutations were WT for K-Ras. Separation of the cell lines according to Ras/BRAF mutation status resulted in a tendency for Ras/BRAF WT cell lines to be more sensitive to cetuximab compared with Ras/BRAF mutant lines, although this effect was not statistically significant (22.6 ± 5.4% versus 40.0 ± 9.4% growth inhibition for Ras/BRAF mutant versus WT cell lines, respectively; $P = 0.11$; Fig. 5*A*).

Collective consideration of PIK3CA/PTEN and RAS/BRAF mutation status further predicts cetuximab response. Notably, our mutation screening analysis showed that, whereas 9 of 16 cell lines (56%) with Ras/BRAF mutations harbored a synchronous PIK3CA/PTEN mutation, 9 of the 10 cell lines (90%) harboring PIK3CA/PTEN mutations contained synchronous Ras/BRAF mutations (Table 1). This finding is consistent with previous reports indicating significant overlap between the presence of PIK3CA and K-Ras/BRAF mutations within the same colorectal tumor (19, 33). Importantly, separation of cell lines that were both PIK3CA mutant/PTEN null and Ras/BRAF mutant, versus those that were not, further discriminated between cetuximab-sensitive and cetuximab-resistant cell lines, with PIK3CA mutant/PTEN null;Ras/BRAF mutant lines (10.8 ± 4.3% growth inhibition) being significantly more resistant to cetuximab than cell lines that did not harbor mutations in both of these pathways (38.8 ± 5.9% growth inhibition, $P = 0.002$; Fig. 5*A*).

Discussion

The development and approval of novel therapies, including the monoclonal antibodies bevacizumab (anti-vascular endothelial growth factor; Avastin) and cetuximab (anti-EGFR), have increased median survival of patients with metastatic colon cancer to ~24 months (34, 35).

Cetuximab was initially approved for use in colorectal cancer patients with EGFR overexpression; however, recent retrospective clinical reports found no evidence for limiting cetuximab therapy to patients with EGFR-overexpressing tumors (8, 9). Similarly, our present findings failed to show an association between EGFR expression and cetuximab response in colon cancer cell lines, indicating that factors other than EGFR expression are primarily responsible for determining response to this agent.

In addition to EGFR expression, a link between EGFR amplification and response to anti-EGFR monoclonal antibody–based treatment has been shown (23). For example, the Difi colon cancer cell line, which has high-level EGFR amplification, is highly sensitive to cetuximab, undergoing both growth arrest and apoptosis at cetuximab concentrations several orders of magnitude lower than those required for response in other cell lines (6, 36). Lievre et al. also described a complete response in a patient with high-level EGFR amplification (20 copies per nucleus; ref. 22). EGFR amplification, however, is a rare event in colon cancer, occurring at a frequency of <1% of cases (37–39). Consistent with this finding,

Table 1. Correlation of mutation status of cell lines and response to cetuximab

Cell line	% Inhibition relative to control (mean ± SE)	Mutant K-Ras Ex 2	Mutant BRAF exon 15	Mutant PIK3CA exon 9	Mutant PIK3CA exon 20	Mutant total PIK3CA	PTEN null	Mutant RAS/BRAF	Mutant PIK3CA/PTEN	RAS/BRAF and PIK3CA/PTEN mutant
LIM1215*	79.6 ± 3.5									
GEO †	68.7 ± 1.6	+						+		
SW403 ‡	66.0 ± 4.9	+						+		
CAC02 ‡	47.7 ± 4.3									
SW948 ‡	42.7 ± 1.4			+		+			+	
HT29 ‡	36.5 ± 8.2		+					+		
SKCO1 ‡	33.9 ± 3.5	+						+		
RW2982*	33.0 ± 1.3									
HCT8*	27.1 ± 4.0	+		+		+		+	+	+
DLD †	24.9 ± 7.4	+		+		+		+	+	+
SW480 †	23.7 ± 3.0	+						+		
SW837 ‡	21.8 ± 8.5	+						+		
SW48 ‡	21.8 ± 1.2									
RKO ‡	21.2 ± 6.9		+		+	+		+	+	+
HCC2998 ‡,§	15.2 ± 3.3									
SW620 ‡	14.5 ± 2.2	+						+		
LS174T ‡	13.0 ± 3.9	+			+	+		+	+	+
T84 ‡	11.2 ± 9.2	+		+		+		+	+	+
KM12 ‡	7.1 ± 9.1	+					+	+	+	+
HCT15 ‡	4.5 ± 6.5	+		+		+		+	+	+
LIM2405*	2.0 ± 2.2		+				+	+	+	+
HCT116 ‡	−14.1 ± 1.3	+			+	+		+	+	+

NOTE: Sensitivity of the cell line panel to cetuximab treatment and mutation status of PIK3CA, BRAF, and K-Ras and expression status of PTEN in colon cancer cell line panel. Values shown are mean ± SE at the 20 μg/mL dose of cetuximab, 72 h posttreatment ($n = 3–5$).

* Present study.

† Ras/BRAF from cosmic; PIK3CA by present study.

‡ From cosmic database, http://www.sanger.ac.uk/genetics/CGP/cosmic/.

§ Mutations in p85α (49) and in codon 146 of K-Ras (48) have been reported in this cell line.

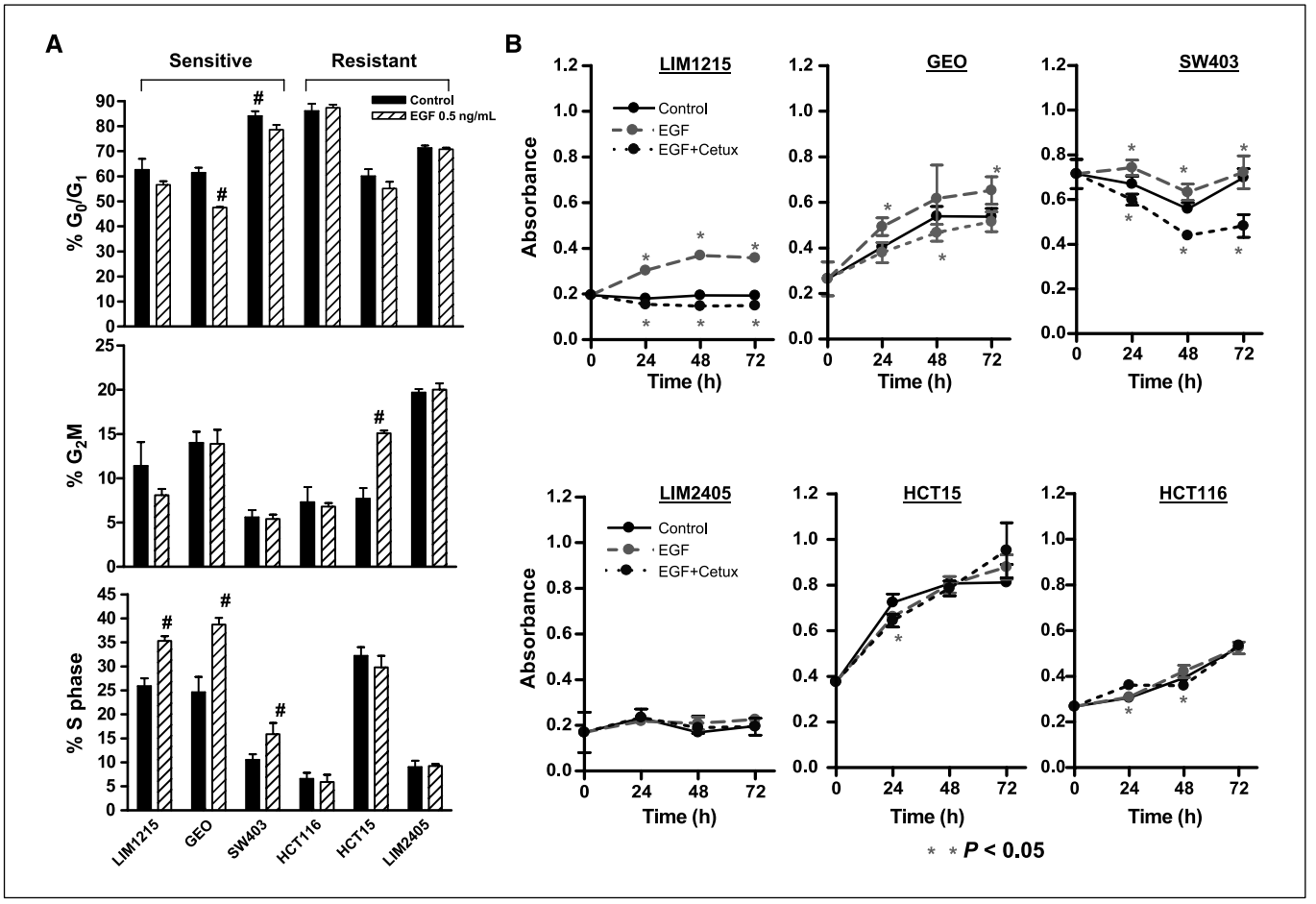

Figure 4. EGF selectively stimulates cell growth in cetuximab-sensitive cell lines. *A,* effect of EGF treatment on cell cycle progression. Cells were treated for 24 h with 0.5 ng/mL EGF, and cell cycle distribution was determined by propidium iodide staining and FACS analysis. *Points,* mean from a representative experiment; *bars,* SE; #, $P < 0.05$. Experiments were repeated in three separate times. *B,* the three most cetuximab-sensitive and cetuximab-resistant cell lines were treated for 24 to 72 h with 0.5 ng/mL EGF or EGF + cetuximab (20 μg/mL) or left untreated (control). Cell growth was assayed by MTT assay. Values shown are mean ± SE from a representative experiment; #, $P < 0.05$. Experiments were repeated three separate times.

examination of EGFR amplification status in cetuximab-sensitive and cetuximab-resistant cell lines in the present study failed to identify any lines with EGFR amplification. These findings shows that, whereas rare tumors and cell lines with EGFR amplification are highly sensitive to cetuximab, more modest, cytostatic responses to this agent are obtained in the absence of EGFR amplification.

Two of the cell lines identified as cetuximab-sensitive, LIM1215 and SW403, have previously been shown to be dependent upon activation of EGFR in an autocrine manner for their growth (40, 41). A previous study also showed a link between the proliferative response of colon adenocarcinoma cell lines to EGF and response to EGFR inhibition (42). We confirmed and extended these observations by demonstrating that cetuximab-sensitive cell lines were more sensitive to EGF-induced growth promotion than cetuximab-resistant lines. Importantly, the growth stimulatory effect of EGF could be completely inhibited by pretreatment with cetuximab. These findings indicate that a subset of colon tumors that are dependent upon ligand activation of EGFR for their growth exists, and it is these cell lines that are growth inhibited by blockade of ligand binding to the EGFR.

As recently shown, tumor cells can lose their dependence on growth factors via mutation-driven constitutive activation of signaling pathways downstream of growth factor receptors, specifically the PI3K and Ras/MAPK pathways (43). We observed that stratification of cell lines according to PIK3CA/PTEN expression mutation status identified a significant difference in cetuximab response, with PIK3CA mutant/PTEN null lines being consistently more resistant to this agent compared with WT lines. This finding was further confirmed using the HCT116 PIK3CA WT and mutant isogenic cell lines, where increased sensitivity to cetuximab was observed in the PIK3CA WT line. This finding is consistent with the reported observation that HCT116 PIK3CA WT cells are more dependent on serum-derived growth factors for their growth and are more responsive to EGF ligand–induced signaling compared with the mutant line (26). Collectively, these findings imply that colon cancer cell lines which acquire mutations that result in constitutive activation of the PI3K pathway have a diminished dependence on canonical EGFR ligand–induced signaling for their growth and are, therefore, more resistant to EGFR-targeted therapies.

Two prior studies failed to observe a link between PIK3CA mutation status and cetuximab response in patients with colon cancer (22, 23). However, in both of these studies, PTEN mutation status of the tumors was not examined, and very few patients with PIK3CA mutations, 3 of 31 (23) and 2 of 30 (22), were identified. Notably, whereas the low PIK3CA mutation frequency may have

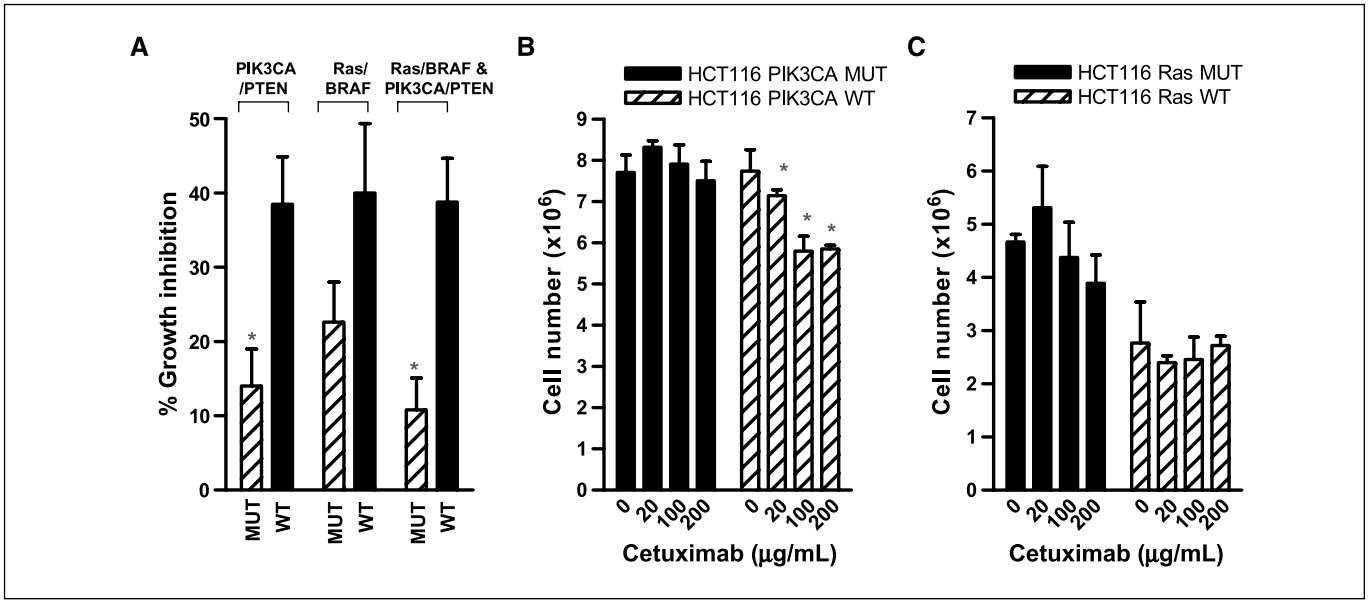

Figure 5. Mutation status and response to cetuximab *in vitro. A,* cetuximab response in the 22–colon cancer cell line panel separated according to PIK3CA mutation/PTEN expression status (*, $P = 0.008$), Ras/BRAF mutation status ($P = 0.11$), or according to presence or absence of synchronous mutations in the PIK3CA/PTEN and the K-Ras/BRAF pathways (*, $P = 0.002$). *B,* cetuximab response in isogenic PIK3CA mutant and WT HCT116 cell lines. Cells were serum starved overnight, then treated with 20 or 100 μg/mL cetuximab for 24 h in medium containing 0.5% serum. Differential sensitivity was assessed by direct counting of cell number. *C,* cetuximab response in isogenic Ras mutant and WT HCT116 cells. Cetuximab response was determined 24 h posttreatment by counting cell number.

precluded the overall ability of these studies to detect differences in cetuximab response between PIK3CA WT and mutant tumors, four of the five patients with PIK3CA mutations did not respond to cetuximab. Furthermore, the single patient with a PIK3CA mutation that did respond had a tumor with low-level EGFR amplification. Collectively considered therefore, the current data available in patient samples are relatively consistent with the present findings, although the need for validation in a larger patient study clearly remains.

Consistent with the present findings, Frattini et al. recently reported that colon tumors with loss of PTEN expression have significantly reduced response to cetuximab (44). Likewise, breast cancers with either activating mutations in PIK3CA or with loss of PTEN expression respond poorly to treatment with the Her2/Neu targeting antibody, trastuzumab (45). Collectively, these studies provide additional clinical evidence that the mutation status of the PI3K signaling pathway should be considered before treatment with EGFR family antagonists.

Several (20–22, 46), although not all, studies (23) that have examined the link between Ras mutation status and cetuximab response have shown that patients with tumors WT for both K-Ras and BRAF have improved response to cetuximab. It is notable, however, that, in some of these studies, patients with mutant Ras tumors, who showed clinical response to cetuximab treatment, were identified (20). In the present study, separation of cell lines according to Ras and/or BRAF mutation status did not stratify cell lines according to cetuximab response, although a clear tendency for Ras/BRAF mutant lines to be more resistant to cetuximab was observed ($P = 0.11$). We also observed that two Ras mutant cell lines, GEO and SW403, showed significant response to cetuximab.

Whereas it remains to be clarified whether determination of Ras/BRAF mutation status alone is sufficient to stratify patients for cetuximab treatment (20), we observed that the six most cetuximab-resistant cell lines harbored mutations or loss of expression in both the PIK3CA/PTEN and Ras/BRAF pathways. This finding is consistent with previous reports indicating the coexistence of Ras and PIK3CA mutations within the same tumor (19, 33). Importantly, collective consideration of PIK3CA mutation/PTEN expression and Ras/BRAF mutation status provided the most robust determinant of cetuximab response, with cell lines harboring mutations (or loss of PTEN expression) in both of these pathways being highly resistant to cetuximab. In terms of therapeutic implications, these findings suggest that patients whose tumors harbor simultaneous PIK3CA mutations/PTEN loss and Ras/BRAF mutations are unlikely to benefit from cetuximab treatment. Instead, these patients may be suitable candidates for treatment with newer targeted drugs currently in clinical trial, which inhibit signaling mediators further downstream, including PI3K, AKT, or mTOR inhibitors and Ras, Raf, or MEK inhibitors.

Importantly, although cell lines with PIK3CA mutation/PTEN loss and Ras/BRAF mutation were consistently resistant to cetuximab, not all cell lines WT at the four loci tested, were sensitive to cetuximab. A possible mechanism of resistance to cetuximab of these cell lines may be the existence of alternate mutations in the Ras/BRAF and or PIK3CA/PTEN pathway other than those screened for in the present analysis. For example, with regards to the PI3K pathway, less frequently occurring mutations have been described in exons 1 and 2 of PIK3CA which encode the p85 interacting domain (18). Mutations in p85α (47), PDK1, AKT2, PAK4, and INSRR (19), as well as amplifications in AKT2 and IRS2 (19), have also been described in colon tumors, as have less frequently occurring mutations in codon 146 (A146T) of the K-Ras gene (48). In this regard, it is notable that mutations in both codon 146 of K-Ras and in p85α have been reported in the relatively resistant HCC2998 cell line (48, 49), which is WT at the four hotspot loci examined in the present analysis. Comprehensive screening of all known components of the PI3K and Ras signaling pathways may therefore be required to further improve prediction of cetuximab response.

In addition to inhibition of EGFR signaling, cetuximab can stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity *in vivo* (50), which may contribute to its antitumor activity. As our current *in vitro* model does not assess the role of ADCC, additional validation of these findings in patient samples is clearly required.

In conclusion, this study shows that cell lines responsive to canonical EGFR signaling-mediated growth are also responsive to cetuximab. We observed that cell lines mutant for PIK3CA/PTEN null are significantly more resistant to cetuximab compared with PIK3CA/PTEN WT lines. Furthermore, cell lines with both constitutively active PIK3CA and Ras/BRAF signaling were highly refractory to cetuximab. Determination of the mutation status of signaling mediators downstream of EGFR may help stratify patients likely to benefit from cetuximab.

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