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 G2-M 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 = 0.0008). 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.


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 82% 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...
therefore that colon tumors with constitutively activated down- 
stream 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 signifi-
cantly 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 HCT16 
K-Ras and PIK3CA WT and mutant cells were generously provided by the 
Sassuaku and Vogelstein/Véulescu 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 experi-
ments, 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 × l) / 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, ATGCTCTACAACCCCAAC; R, GCTGCTGCACTCTTACAC). 
Results were expressed relative to glyceraldehyde-3-phosphate dehydrogenase 
(primers: F, TCGGATCTCAAGC-GATTTGG; R, GAATTTGCCATGGTGGAAT).

Determination of EGFR gene copy number by fluorescence in situ 
hybridization. EGFR copy number was ascertained in colon-carcinoma treated-cells 
by standard cytogenetic methods using 0.075 mol/L KCI 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 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, AGGCCGTCTGAAATGACT-
GATAA and R, CTGATCAAAGATGTTCTGCAC. Primers used were F, 
AACACATTCTAAGGCCCCAA and R, GAAATGCTTCCAAATATTCGTT 
for amplification of exon 15 of BRAF; F, GCTTTTTCTGAAATCCTGCT 
and R, CTGAGATACGAAAAATTCTAGT for exon 9 of PIK3CA; and F, 
CATTTGCTCAAAGATGMCACA and R, TACTCAGAGCCTTGCTC (for 
codon 1023 mutation) and F, ACATTTGAAAGACCTAGCC and R, 
CAATTTCTGTCAAATCGCT (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, cetu-
xicimab inhibited growth of GEO cells by 75% (205 ± 36 mm^3 
compared with 854 ± 201 mm^3 in cetuximab-treated and control 
mouse, 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 mouse, respectively; Fig. 1B).

4 http://www.sanger.ac.uk/genetics/CGP/cosmic/
Comparable sensitivity profiles of colon cancer cells to cetuximab and erlotinib. 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 G0-G1 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. 2A–C). Consistent with the MTT data, an increase in the percentage of cells in G0-G1 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 G2-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. 2D), 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. 2D).

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. 3A, 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 ($R^2 = 0.067$, $P = 0.244$), no correlation between basal EGFR protein expression and cetuximab response was observed ($R = -0.36$, $R^2 = 0.13$, $P = 0.099$; Fig. 3B).

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. 3C, 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. 4A). 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. 4B). 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. 4B).

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 PIP3 to PIP2, 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...
cetuximab, harbors a truncating mutation in PTEN (Wellcome Trust Sanger Institute Cancer Genome Project). 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. 5A).

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. 5B). 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. 5C).

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

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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. 5.4).

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 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. 5.4).

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

<table>
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<th>Cell line</th>
<th>% Inhibition relative to control (mean ± SE)</th>
<th>Mutant K-Ras Ex 2</th>
<th>Mutant BRAF exon 15</th>
<th>Mutant PIK3CA exon 9</th>
<th>Mutant PIK3CA exon 20</th>
<th>Mutant total PIK3CA</th>
<th>PTEN null</th>
<th>Mutant RAS/BRAF</th>
<th>Mutant PIK3CA/PTEN</th>
<th>RAS/BRAF and PIK3CA/PTEN mutant</th>
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<tr>
<td>LIM1215*</td>
<td>79.6 ± 3.5</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>GEO</td>
<td>68.7 ± 1.6</td>
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<td>+</td>
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<tr>
<td>SW408</td>
<td>66.0 ± 4.9</td>
<td>+</td>
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<td>42.7 ± 1.4</td>
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<td>HT29</td>
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<td>HCT8*</td>
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<td>DLD</td>
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<td>HCC2998*</td>
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<td>+</td>
<td>+</td>
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<td>LS174T*</td>
<td>13.0 ± 3.9</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>T84</td>
<td>11.2 ± 9.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>KM12</td>
<td>7.1 ± 9.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HCT15</td>
<td>4.5 ± 6.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LIM2405*</td>
<td>2.0 ± 2.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HCT116</td>
<td>-14.1 ± 1.3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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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.

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.
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 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.
precluded the overall ability of these studies to detect differences in 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.
In addition to inhibition of EGFR signaling, cetuximab can stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity in vivo, 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.

Acknowledgments

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References

Correction: Prediction of Response to Cetuximab

In the article on prediction of response to cetuximab in the March 15, 2008 issue of Cancer Research (1), the correct name of the twelfth author is Roman Perez-Soler.

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

Minaxi Jhawer, Sanjay Goel, Andrew J. Wilson, et al.


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