Oncogenic Activation of the RAS/RAF Signaling Pathway Impairs the Response of Metastatic Colorectal Cancers to Anti–Epidermal Growth Factor Receptor Antibody Therapies

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Abstract

Monoclonal antibodies (mAbs) against the extracellular domain of the epidermal growth factor receptor (EGFR) have been introduced for the treatment of metastatic colorectal cancer (mCRC). We have reported recently that increased copy number of the *EGFR* can predict response to anti-EGFR mAbs and that patients might be selected for treatment based on EGFR copy number. Here, we show that mutations activating the RAS/RAF signaling pathway are also predictive and prognostic indicators in mCRC patients, being inversely correlated with response to anti-EGFR mAbs. In cellular models of CRCs, activation of the RAS signaling pathway by introduction of an activated *K-RAS* allele ($Gly^{12}Val$) impairs the therapeutic effect of anti-EGFR mAbs. In cancer cells carrying constitutively active RAS, the pharmacologic inhibition of the mitogen-activated protein kinase (MAPK) signaling cascade improves anti-EGFR treatment based on mAbs. These results have implications for the identification of patients who are likely to respond to anti-EGFR treatment. They also provide the rationale for combination therapies, targeted simultaneously to the EGFR and RAS/RAF/MAPK signaling pathways in CRC patients. [Cancer Res 2007;67(6):2643–8]

Introduction

Cetuximab and panitumumab are two monoclonal antibodies (mAbs) directed against the epidermal growth factor receptor (EGFR; ref. 1). Both the chimeric antibody cetuximab and the fully human antibody panitumumab have shown remarkable clinical activity in ~10% of patients with chemotherapy-resistant metastatic colorectal cancers (mCRC; refs. 2–5). We have reported previously that most patients with mCRC who achieve tumor shrinkage from treatment with anti-EGFR mAbs exhibit increased *EGFR* gene copy number (2). In these patients, the tumor growth is likely to be driven predominantly by the EGFR pathway (6, 7). A fraction of patients with increased *EGFR* gene copy number respond, whereas patients with normal *EGFR* gene copy status are unlikely to respond to the therapy (8). We did not observe a statistical association between clinical response and the mutational status of the *EGFR* gene (2). This finding is in accordance with other studies, indicating that *EGFR* mutations are an infrequent event in CRC (9).

Regardless of their genetic status, after a variable period, CRCs develop resistance to the mAbs, thus severely impairing their therapeutic potential. For these reasons, it is a clear priority to understand the molecular and cellular basis of primary and acquired resistance to anti-EGFR mAbs, cetuximab and panitumumab. We hypothesized that the lack of primary response or the acquired resistance to anti-EGFR mAbs may be due to constitutive activation of the signaling pathways downstream of the receptor. Our hypothesis is based on the concept that the genes mutated in cancer work in organized pathways. Mutations within a single pathway are often "mutually exclusive," as predicted if the functional effect of each mutation was similar (10, 11). Consequently, only one of the genes involved in a given pathway is generally mutated in any single tumor. This implies that pathways rather than single genes should be the focus of studies aimed at dissecting the molecular basis of targeted therapies. It also suggests that once the "culprit" pathway governing the oncogenic property of a cancer is identified, all the players involved in that pathway (and not only a specific gene) can become a suitable therapeutic target.

Previous work has shown that two main signaling pathways are triggered by activation of the EGFR (12). The first is initiated by the small G-protein RAS, which in concert with the protein kinase RAF activates the mitogen-activated protein kinase (MAPK) cascade. The second involves the lipid kinase phosphatidylinositol 3-kinase that triggers activation of the PDK1-AKT signaling machinery (13).

It has been reported recently that *K-RAS* mutations negatively correlate with the response to the mAb cetuximab (14). In our previous report, the mutational status of the EGFR signaling effectors (K-RAS, BRAF, or PIK3CA) was not statistically associated with the response, although the *K-RAS* mutations seemed to be slightly more frequent in nonresponsive patients (2).

To assess whether activation of these signaling pathways could be connected to the response to anti-EGFR mAbs, we used two complementary approaches. First, genetic analysis was used to identify which members of the pathway were oncogenically activated in mCRCs from patients that had received the anti-EGFR mAbs. Second, CRC cell models were used to assess whether activation of effectors downstream the EGFR pathway affected the response to anti-EGFR treatment.

Materials and Methods

Patient characteristics and treatments. We assessed tumors from 48 patients with mCRC enrolled into clinical trials of panitumumab (Amgen, Thousand Oaks, CA) or cetuximab (Erbitux, Merck, Milan, Italy) for

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treatment of EGFR-expressing mCRC at Ospedale Niguarda Ca' Granda (Milan, Italy). The retrospective analysis of tumor DNA sequences of *K-RAS* and *BRAF* was approved by the Ethics Committee of Ospedale Niguarda Ca' Granda. Patients were selected based on the availability of sufficient tumor tissue. All patients had EGFR-expressing mCRC and 1% or more malignant cells that stained for EGFR on immunohistochemical analysis with DAKO EGFR PharmDX kit (DakoCytomation, Glostrup, Denmark) done in the central laboratory of each clinical trial. Demographics of patients, treatment and line of treatment received, objective responses, and durations of response are summarized in Table 1. In detail, 12 (25.0%) patients received cetuximab monotherapy, 25 (52.1%) received panitumumab monotherapy, and 11 (22.9%) received cetuximab plus irinotecan–based chemotherapy (Camptò, Aventis, Milan, Italy). Cetuximab plus irinotecan were administered to patients with proven refractoriness to irinotecan, defined as documented disease progression during, or within, 3 months of receiving an irinotecan regimen. Tumor response was assessed with computed tomography or magnetic resonance imaging by use of Response Evaluation Criteria in Solid Tumors criteria according to clinical protocols by radiologist investigators at Niguarda Ca' Granda Hospital. All of the patients progressed after treatment and all but one were assessable for progression-free survival analysis.

Mutational analysis. DNA was extracted from paraffin-embedded samples. For each patient, 10-μm sections were prepared. An additional representative 2-μm section was deparaffinized, stained with H&E, and analyzed for detailed morphology. Regions displaying tumor tissues were marked and the tissue was extracted as described previously (2). Exon-specific and sequencing primers were designed using Primer3 software (15) and synthesized by Invitrogen (Milan, Italy) as previously described (2). Conditions to amplify exon-specific regions by PCR from tumor genomic DNA and to identify mutations have been described previously (16). PCR was carried out in a volume of 20 μL using a touchdown PCR program as described previously (2). Purified PCR products were sequenced using BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Milan, Italy) and analyzed with a 3730 ABI capillary electrophoresis system. Mutational analysis was carried out as described previously. Tumor tissue from patient 29 was limited in quantity and mutational analysis was therefore not technically possible for all exons.

CRIB assays. The RAF-CRIB domain pull-down experiment on cells transfected with mutated K-RAS or the control vector was carried out as described previously by Taylor and Shalloway (17). Cells were lysed in a lysis buffer containing 25 mmol/L HEPES (pH 7.3), 0.3 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 mmol/L DTT, 0.5% Triton X-100, and 5% glycerol containing 0.5 mmol/L phenylmethylsulfonyl fluoride and 5 μg/mL each of aprotinin and pepstatin. Lysates were then incubated with slurry beads previously linked to glutathione *S*-transferase (GST)-RAF CRIB domain and GST alone for 2 h at 4°C, washed three times in lysis buffer, resuspended, and loaded on a 12% acrylamide gel. Western blots were carried out as described previously (2).

Cell culture and transfections. CRC cell lines were obtained from the American Type Culture Collection (Manassas, VA) repository with the exception of the DiFi cells that were supplied by Jose Baselga (Vall d'Hebron University, Barcelona, Spain). Cells were grown in medium supplemented with 10% FCS and antibiotics. The constitutively active K-RAS expression vector used was pDCR-H-RasV12, kindly provided by Letizia Lanzetti (Torino Medical School, Candiolo, Torino, Italy). Empty vectors were used as controls for the transfections. Cells were transiently transfected using LipofectAMINE as suggested by the manufacturer.

Proliferation inhibition assay and Western blotting. For cell viability assays, cells were grown in medium supplemented with 2% FCS and incubated for 5 days with increasing concentrations of cetuximab (Komtur Pharmaceuticals, Freiburg, Germany) and/or PD98059 (Calbiochem, Milan, Italy). CRC cells were seeded in 96-well plates, and at the end of the drug incubation period, a tetrazolium salt–based reagent (CellTiter96 Aqueous One Solution, Promega, Milan, Italy) was added to each well according to the instructions provided by the manufacturer. After an incubation of 2 h, absorbance was read at 490 nm on a Beckman Coulter (Milan, Italy) DTX 880 plate reader. Values for control cells were considered as 100% viability. The triplicate values were all within 5% and the mean values were calculated and plotted with error bars representing the SD of triplicate samples from three independent experiments. Western blotting was done as described (2).

Statistics. Data have been analyzed using Stata 9.1 (Stata Corp., College Station, TX); all significance levels were set at $P < 0.05$. Qualitative comparisons of objective response to therapy (progressive disease, stable disease, and partial response) and gene mutations as predictors were done by the Fisher's exact test to check possible significance; the response to therapy was then analyzed by logistic regression using the presence of gene mutations as regressors. All logistic analyses were checked by means of pseudo R^2 value and post tested with Hosmer and Lemeshow goodness-of-fit test followed by receiver operating characteristic analysis to verify sensitivity, specificity, and positive/negative predictive values of each model. The time-to-progression (TTP) analysis was done after Kaplan-Meier and then survivor curves were compared by means of log-rank test. TTP was defined as the time from random assignment of each protocol (see patients characteristics and treatments) until first documented tumor progression or death.

Results

Mutational profiling of the EGFR signaling pathways in patients treated with cetuximab or panitumumab. The mutational status of *K-RAS* (exon 2) and *BRAF* (exons 15 and 21) was assessed in a series of 48 patients who had received either cetuximab or panitumumab (Table 1 and 2). Informative sequences were obtained for all cases with the exception of one patient (patient 29), for which the mutational status of *BRAF* could not be ascertained due to insufficient starting material. *K-RAS* mutations were detected in 16 of the 48 (33.3%) tumors, and *BRAF* mutations were detected in 6 of the 48 (12.5%) tumors (Table 2). The most frequent *K-RAS* alterations in our samples were single amino acid substitutions in codon 12 [10 of the 16 (62.5%) mutated tumors]. Single amino acid substitutions were also detected in codon 13, but less frequently [6 of 16 (37.5%)]. The only *BRAF* mutation found in our samples was the previously described V600E substitution that was detected in six patients. Taken together, 45.8% of the analyzed tumors carried a mutation either in *K-RAS* or *BRAF* genes. *K-RAS* and *BRAF* mutations were mutually exclusive exactly as expected if they were acting in the same signaling pathway.

RAS/RAF mutations are negatively associated with response to cetuximab or panitumumab. Next, we attempted to assess whether the mutational status of *K-RAS* or *BRAF* was associated with the clinical response to anti-EGFR mAbs (Table 2). The presence of *K-RAS* mutations was not significantly linked to objective response to therapy, with a trend toward a negative association with response (1 of 11 mutations versus 15 of 37 mutations for responders versus nonresponders; $P = 0.073$). *BRAF* mutations alone were also not significantly associated with objective response to therapy ($P = 0.312$). Importantly, however, the presence of K-RAS and/or *BRAF* mutations was negatively associated with partial response ($P = 0.005$) and the logistic regression confirmed this association (odds ratio, 0.071; 95% confidence interval, 0.08–0.619; $P = 0.017$). These results suggest that patients carrying either *K-RAS* or *BRAF* mutations are less likely to achieve partial response to therapy with anti-EGFR mAbs. Our data also indicate that signaling pathways rather than single genes should be the focus of genetic studies aimed at dissecting the molecular basis of targeted therapies.

***RAS/RAF* mutations negatively correlate with progression-free survival of mCRC patients treated with cetuximab or panitumumab.** Next, we assessed whether in patients treated

Table 1. Demographics of patients, treatment received, and objective response

Patient ID	Age	Sex	Line of treatment for metastatic disease	Anti-EGFR therapy	Tumor response	
					Best response	Duration (wk)
1	59	M	4th	Panitumumab	PR	31
2	62	F	3rd	Panitumumab	PR	23
3	57	M	3rd	Panitumumab	SD	15
4	78	F	4th	Panitumumab	PR	24
5	63	M	3rd	Panitumumab	PR	15
6	71	M	3rd	Panitumumab	SD	32
7	60	M	4th	Panitumumab	SD	24
8	58	M	4th	Panitumumab	PD	NA
9	68	M	4th	Panitumumab	SD	23
10	56	M	2nd	Panitumumab	PD	NA
11	67	F	3rd	Panitumumab	PD	NA
12	54	M	3rd	Panitumumab	PD	NA
13	65	F	4th	Panitumumab	PD	NA
14	57	M	4th	Panitumumab	PD	NA
15	62	F	4th	Panitumumab	PD	NA
16	46	F	3rd	Panitumumab	PD	NA
17	53	F	4th	Panitumumab	PD	NA
18	67	M	3rd	Panitumumab	PD	NA
19	61	M	4th	Panitumumab	PD	NA
20	70	F	4th	Panitumumab	PD	NA
21	63	F	3rd	Panitumumab	SD	15
22	44	M	4th	Panitumumab	SD	16
23	47	F	3rd	Panitumumab	PD	NA
24	52	F	4th	Panitumumab	SD	16
25	53	F	4th	Panitumumab	SD	31
26	50	F	4th	Cetuximab + CT	PR	50
27	58	M	4th	Cetuximab + CT	PR	45
28	68	M	3rd	Cetuximab + CT	PR	47
29	74	M	4th	Cetuximab + CT	SD	23
30	73	M	4th	Cetuximab + CT	PD	NA
31	39	M	4th	Cetuximab + CT	PD	NA
32	54	F	5th	Cetuximab + CT	PD	NA
33	78	M	1st	Cetuximab	PR	24
34	84	M	1st	Cetuximab	PR	17
35	75	F	1st	Cetuximab	SD	60
36	74	M	1st	Cetuximab	SD	25
37	57	M	1st	Cetuximab	PD	NA
38	61	M	1st	Cetuximab	PD	NA
39	71	F	1st	Cetuximab	PD	NA
40	71	M	1st	Cetuximab	PD	NA
41	76	M	1st	Cetuximab	PD	NA
42	75	F	1st	Cetuximab	PD	NA
43	76	M	1st	Cetuximab	PD	NA
44	53	M	4th	Cetuximab	PR	50
45	57	M	2nd	Cetuximab + CT	SD	33
46	55	M	3rd	Cetuximab + CT	SD	25
47	51	M	3rd	Cetuximab + CT	PD	NA
48	59	F	3rd	Cetuximab + CT	PR	—

Abbreviations: M, male; F, female; CT, computed tomography; SD, stable disease; PD, progressive disease; PR, partial response; NA, not applicable; —, data not available.

with anti-EGFR mAbs the oncogenic activation of downstream signaling pathways was associated with time to tumor progression, which is a more compelling evidence of clinical benefit than objective response especially considering biological agents such as mAbs. The TTP analysis showed a significantly worse outcome for subjects bearing a mutated *K-RAS* allele in their tumors compared with those carrying wild-type (WT) *K-RAS* ($P = 0.0443$; Fig. 1*A*). The same was not observed for CRC patients carrying the BRAF mutation ($P = 0.5369$), probably due to the limited number of tumors carrying these mutations compared with those

Table 2. K-RAS and BRAF mutations and response in mCRC treated with anti-EGFR mAbs

Patient ID	Sequencing analysis		Best response
	K-RAS	BRAF	
1	WT	WT	PR
2	G13D	WT	PR
3	G12D	WT	SD
4	WT	WT	PR
5	WT	WT	PR
6	G12V	WT	SD
7	WT	V600E	SD
8	WT	WT	PD
9	WT	V600E	SD
10	WT	WT	PD
11	G13D	WT	PD
12	WT	WT	PD
13	WT	V600E	PD
14	G12V	WT	PD
15	WT	WT	PD
16	G12V	WT	PD
17	G12D	WT	PD
18	WT	V600E	PD
19	WT	WT	PD
20	G13A	WT	PD
21	G12V	WT	SD
22	WT	V600E	SD
23	WT	V600E	PD
24	G13D	WT	SD
25	WT	WT	SD
26	WT	WT	PR
27	WT	WT	PR
28	WT	WT	PR
29	WT	—	SD
30	G12V	WT	PD
31	WT	WT	PD
32	G13V	WT	PD
33	WT	WT	PR
34	WT	WT	PR
35	WT	WT	SD
36	G12D	WT	SD
37	WT	WT	PD
38	WT	WT	PD
39	G12S	WT	PD
40	WT	WT	PD
41	G12D	WT	PD
42	WT	WT	PD
43	WT	WT	PD
44	WT	WT	PR
45	WT	WT	SD
46	WT	WT	SD
47	G13D	WT	PD
48	WT	WT	PR

carrying the *K-RAS* mutations. However, the presence of either one or other mutation was still significant (P = 0.0259; Fig. 1*B*). This could imply that constitutive activation of the RAS/RAF signaling pathway impairs the response to therapy based on anti-EGFR antibodies and is in good agreement with what observed in other models, such as the response of lung cancer to gefitinib or erlotinib (8, 18).

Oncogenic activation of the RAS signaling pathway impairs the response of CRC cells to cetuximab. The results we obtained from the genetic profiling of CRCs suggest that the presence of mutated *K-RAS* negatively interferes with the clinical response to anti-EGFR mAbs, such as cetuximab and panitumumab. They also indicate that the acquisition of a secondary *K-RAS* mutation (or an equivalent event activating the RAS/RAF pathway) might be a mechanism by which tumor cells initially responsive to anti-EGFR mAbs become resistant to this therapeutic regimen. To test this hypothesis, we used cellular models of CRCs. Specifically, we took advantage of a naturally occurring CRC cell line (DiFi) carrying a 20-fold amplification of the *EGFR* gene (2). We have shown previously that the proliferation of DiFi cells is inhibited by nanomolar concentration of cetuximab; therefore, these cells represent a suitable model to understand the molecular and cellular basis of sensitivity to anti-EGFR mAb therapy (2). Furthermore, our mutational analysis of *K-RAS* and *BRAF* indicated that both genes were WT in DiFi cells (data not shown), thus allowing further functional experiments.

An activated allele of *K-RAS* ($Gly^{12}Val$) was then introduced into DiFi cells alongside with the corresponding control vector using lipid-mediated plasmid DNA transfection. The transfection efficacy was verified using a plasmid encoding for the *GFP* gene. To assess whether we had successfully expressed an active RAS protein into transfected DiFi cells, we measured the amount of RAS bound to GTP. To this end, we did a RAF-CRIB domain pull-down experiment on cells transfected with mutated *K-RAS* or the control vector (see details in Materials and Methods). The results clearly indicated that cells transfected with the *K-RAS* ($Gly^{12}Val$) plasmid have a significantly higher amount of RAS protein bound to GTP when

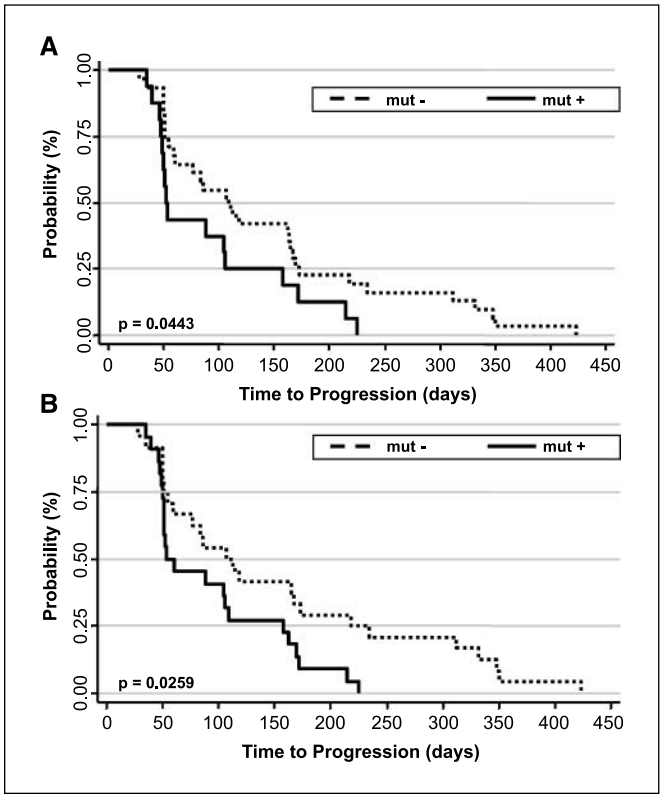

Figure 1. TTP according to the K-RAS (*A*) and K-RAS and/or BRAF (*B*) mutational status.

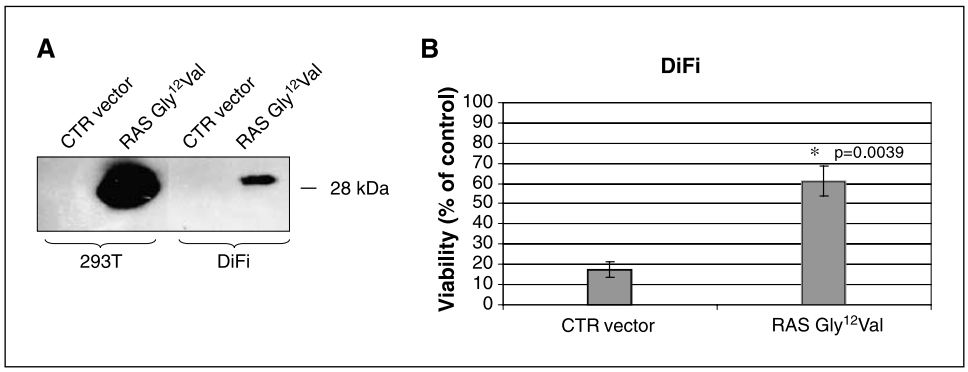

Figure 2. Activated K-RAS confers resistance to cetuximab in DiFi cell line. *A,* cells transfected with either empty vector (*CTR vector*) or RAS $Gly^{12}Val$ were lysed and subjected to a CRIB pull-down assay to check for RAS activity. *B,* DiFi cells were transfected with either empty vector (*CTR vector*) or RAS $Gly^{12}Val$ and then subjected to cetuximab treatment. Several concentrations, ranking from 5 to 20 nmol/L, were tested. Here, we show the results obtained with 20 nmol/L concentration. The graph shows the percentage of survival of the treated cells at day 9 posttransfection ($P = 0.0039$). The experiment was repeated two independent times and each time produced comparable results and *P* values.

compared with those transfected with the control vector (Fig. 2*A*). We then treated DiFi cells expressing mutated and WT *RAS* with increasing concentrations of cetuximab (from 5–20 nmol/L) and measured the percentage of cell viability. We found that cells expressing the activated RAS (*K-RAS* $Gly^{12}Val$) were less sensitive to the drug as indicated by their markedly increased survival upon treatment with cetuximab (Fig. 2*B*). The experiment was repeated twice and each time we obtained comparable results and *P* values.

These results indicate that the presence of oncogenically active K-RAS (such as the $Gly^{12}Val$ mutant) impairs the therapeutic potential of cetuximab in CRC cells. They also suggest that cancers responsive to anti-EGFR mAbs could become resistant to this drug by acquiring a secondary genetic lesion that triggers the constitutive activation of the RAS/RAF/MAPK signaling pathway.

Effect of combinatorial therapies simultaneously targeting the EGFR and the RAS/RAF/MAPK signaling pathways in CRC cells. Our results suggest that constitutive oncogenic activation of the RAS/RAF signaling pathway significantly impairs the therapeutic potential of mAbs (such as cetuximab) aimed at targeting the EGFR in CRCs. It is therefore tempting to speculate that the concomitant blockade of both the EGFR and the RAS signaling could be effective in inhibiting the proliferation of cancer cells. Several inhibitors targeting the RAS signaling pathway have been developed (19, 20). Among them, the MAPK/extracellular

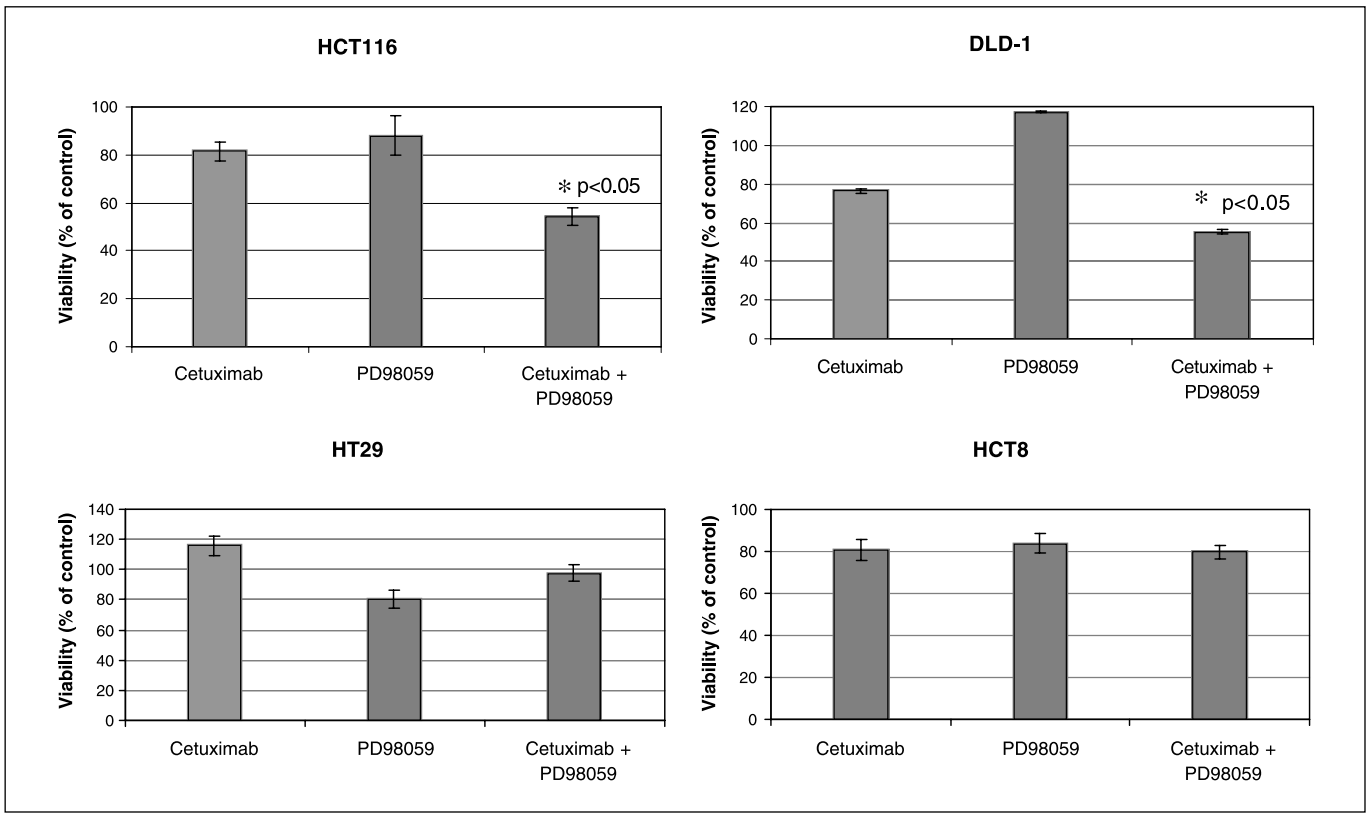

Figure 3. Simultaneous targeting of the EGFR and the RAS/RAF/MAPK signaling pathways in colorectal cancer cells. Survival of mutated (HCT116 and DLD-1) and WT (HT29 and HCT8) *K-RAS* cancer cells treated with cetuximab (6.6 μmol/L), the MAPK inhibitor PD98059 (5 μmol/L), and a combination of the two agents.

signal-regulated kinase kinase inhibitors, such as PD0325901 and ARRY-142886, have shown good preclinical activity (20, 21) and have entered recently clinical trials. To assess whether concomitant inhibition of the EGFR and of the RAS signaling pathway could be effective, we took advantage of PD98059, a powerful cell-permeable inhibitor of MAPKs that acts as downstream effector of RAS (22). For these experiments, we selected CRC cell lines that have been found previously to carry either WT (HT29 and HCT8) or mutated (HCT116 and DLD-1) *K-RAS*. To confirm that HCT116 and DLD-1 cells indeed harbor mutated *K-RAS* alleles, we extracted the corresponding genomic DNA and sequenced the *K-RAS* locus. We confirmed that HCT116 and DLD-1 display the same $Gly^{13}Asp$ *K-RAS* mutation (data not shown). The cell lines were treated with increasing concentration of cetuximab and PD98059 and their viability was measured (Fig. 3). These experiments indicate that, in cancer cells harboring oncogenic K-RAS, the simultaneous inhibition of both the EGFR and the RAS downstream signaling effectors can be more effective than targeting either pathway alone.

Discussion

In the last 5 years, cancer therapy has undergone a major revolution characterized by the introduction of targeted drugs that inhibit specific molecules. Among those, the mAbs (cetuximab and panitumumab) targeting EGFR have shown remarkable efficacy in the treatment of mCRCs. Similar to other targeted therapies, anti-EGFR drugs are active only in a fraction of patients and most of them subsequently become resistant to the treatment. Accordingly, two major challenges need to be addressed to optimize the efficacy of anti-EGFR therapies. The first is to identify the genetic alterations associated with the clinical response to anti-EGFR mAbs. The second is the elucidation of the molecular basis for primary or acquired resistance to these drugs.

In the present study, we report that the presence of mutations (most of which are thought to be gain of function) in *K-RAS* and *BRAF* are associated with the lack of response to anti-EGFR mAb treatment in mCRCs patients. It has been reported previously that the $Gly^{12}Val$ *K-RAS* mutation is associated with increased risk of relapse in CRC (23). This implies that the negative association we found with the occurrence of *K-RAS/BRAF* mutations may not only be due to resistance or lack of response to anti-EGFR mAbs. However, the cellular models in which we inserted the $Gly^{12}Val$ mutation indicate that the presence of an active *K-RAS* allele directly affects the responsiveness of CRC cells to anti-EGFR mAb, such as cetuximab.

Our results have several implications. The first is that most patients with CRC carrying mutated *K-RAS* or *BRAF* are not likely to experience significant benefit on either cetuximab or panitumumab treatment. However, the same patients should not be excluded from anti-EGFR mAbs treatment as we found that there are few mCRCs, in which the presence of *K-RAS* mutations is compatible with a clinical response to this therapeutic regimen. The molecular determinants of response in this subset of patients are presently unknown, and this observation therefore warrants further investigations. The second suggestion stemming from our present work is that clinical trials designed to test multitherapies with both anti-EGFR and anti-MAPK inhibitors should be considered for mCRC patients.

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Note added in proof: In the American Type Culture Collection catalog, the colorectal cancer cell line HCT8 is also referred to as HRT-18. The COSMIC database (Catalogue of Somatic Mutations In Cancer, http://www.sanger.ac.uk/genetics/CGP/cosmic/) indicates that the HRT-18 cell line carries the KRAS G13D mutation.

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