Estimating Preclinical Efficacy Targets Utilizing Cetuximab Efficacy in *KRAS* Mutant and Wild-type Colorectal Cancer Models

MARIE PREWETT, RAJIV BASSI, KEREN PAZ, MICHAEL AMATULLI, DHANVANTHRI DEEVI, HUILING LI, SU WANG, LARRY WITTE, SELDA SAMAKOGLU and JAMES R. TONRA

ImClone Systems, Alexandria Center for Science and Technology, New York, NY, U.S.A.

Abstract. *Background: Clinically relevant targets for developmental drug efficacy in animal models of cancer are critical yet understudied parameters. Materials and Methods: Cetuximab, a chimeric antibody to epidermal growth factor receptor (EGFR), was administered to athymic mice bearing subcutaneous tumors established with 13 human colorectal cancer cell lines of varying biomarker status, defined by DNA sequencing and RT-PCR. Results: If tumor growth inhibition is taken as a target, as is commonly done, then in contrast to the clinical situation where KRAS mutation strongly predicts for a lack of clinically meaningful benefit in colorectal cancer patients, cetuximab alone and in combination with irinotecan-based chemotherapy were efficacious in a similar proportion of KRAS wild-type and mutant models. It was only when tumor regression was utilized to define relevant efficacy that cetuximab monotherapy was efficacious in KRAS wild-type, but not mutant models. Adding cytotoxic therapy to cetuximab treatment increased tumor regression frequency in both genotypes to the point that once again the response was similar for KRAS wild-type and mutant models. Conclusion: Our data support shifting the threshold for claiming clinically relevant targeted therapy efficacy in subcutaneous xenograft models towards tumor regression, rather than tumor growth inhibition, focusing on the evaluation of tumor cells that are addicted to the pathways being targeted.*

Over 1.2 million patients worldwide were diagnosed with colorectal cancer in 2008 (1). Despite recent advances in targeted, cytotoxic and combination therapies, approximately 608,000 deaths were attributed to this disease, accounting for 8% of all cancer deaths in 2008 (1). While there is clearly a need for more effective treatment strategies, drug development costs can exceed 800 million dollars (US) per approved drug (2) and many drugs fail in clinical trials after considerable investment of time and resources. For example, from 1990-2006, only 8% of all agents tested in clinical studies in the United States for the treatment of cancer eventually achieved regulatory approval (3). It is therefore critical to better focus resource investment on treatment strategies with a greater probability of success in the clinic. Enhanced preclinical testing offers a means of selecting these strategies.

Preclinical cancer drug development typically begins with *in vitro* selection of agents targeting pathways or functions associated with cancer progression, followed by testing in animal models of cancer. The most frequent model type utilized in these efforts is the subcutaneous xenograft model in which cancer cell lines are injected under the skin of mice to form growing tumors. Satisfactory demonstration of efficacy in animal models is generally required for advancement into clinical trials (4). The threshold levels of efficacy for achieving this preclinical milestone are however not consistent or clear across the industrial and academic communities. Typically the threshold for activity against subcutaneous tumors in mice, indicating that a treatment 'works' and should be advanced into patients is a treatment/control final tumor volume ratio (T/C%) of less than 50% (5-7), with a 95% level of statistical confidence that the candidate therapy has inhibited tumor growth compared to control treatments (4). Yet continued tumor growth in clinical trials, even if slowed to <50% of controls, is not considered a positive treatment response. Moreover, reduction in the rate of tumor growth is not currently considered justification for drug approval (8). This raises the issue whether inhibition of tumor growth in preclinical models, at any level, is a high enough threshold to predict for success in the clinic, and be utilized to prioritize treatment strategies for advancement into clinical testing.

Examples are common in which inhibition of tumor growth has been observed in preclinical cancer models, but clinically

Correspondence to: Marie Prewett, ImClone Systems, a wholly-owned subsidiary of Eli Lilly and Company, Department of Preclinical Pharmacology, Alexandria Center for Science and Technology, 450 First Avenue, New York, NY 10016, U.S.A. Tel: +1 6466385018, e-mail: marie.prewett@imclone.com

Key Words: Cetuximab, colorectal cancer, irinotecan, oxaliplatin, biomarkers.

relevant efficacy was not achieved in patients with comparable treatment regimens. In preclinical pancreatic cancer models, the chimeric epidermal growth factor receptor (EGFR) antibody cetuximab (ERBITUX®), combined with gemcitabine therapy, inhibited orthotopic L3.7pL tumor growth, as well as liver and regional lymph node metastasis (9). Yet in the clinic, cetuximab plus gemcitabine provided only a 0.4 month improvement in median survival time compared to gemcitabine alone (10). Bevacizumab likewise has been reported in numerous publications to be active (inhibits tumor growth) as a monotherapy in subcutaneous xenograft models, yet data from clinical testing in support of the use of bevacizumab as a monotherapy failed to demonstrate improvement in disease-free survival for resected stage II/III colon cancer (11). As a final example, tipifarnib, an oral nonpeptidomimetic farnesyl transferase inhibitor, induced *in vitro* apoptosis in multiple models of mantle cell lymphoma, and suppressed tumor xenograft growth *in vivo* (12). Yet in a phase II trial of tipifarnib in relapsed/refractory mantle cell lymphoma, the response rate was low (13). There are of course examples of treatment strategies that inhibit tumor growth in preclinical models and which have significant therapeutic benefits in patients, including the addition of cetuximab or bevacizumab to cytotoxic therapy (reviewed in 14). However, the examples of strategies that have 'worked' in preclinical models but not in patients have led some to question the value of preclinical cancer models, especially those utilizing human cancer cell lines (15).

To address this concern however, the threshold for claiming the achievement of potentially clinically relevant efficacy in preclinical models first needs to be more specifically studied and defined. Here we have sought to contribute towards the establishment of such thresholds, by utilizing recent clinical findings demonstrating a clear difference in the therapeutic benefits of antibodies targeting EGFR in patients with metastatic colorectal cancer harboring tumors with an activating mutation in *KRAS*, compared to efficacy in patients with tumors expressing wild-type *KRAS* (16). KRAS is downstream of EGFR activation in signaling networks frequently supporting the proliferation and survival of cancer cells (17-19). KRAS constitutive activation therefore is thought to obviate the benefits of targeting EGFR in cancer patients (16).

The role of mutant *KRAS* as a predictor of response to EGFR-targeted antibody therapy in colorectal cancer patients was first demonstrated for panitumumab (20). Subsequently, a phase II trial utilizing cetuximab therapy in 30 patients with metastatic colon carcinoma found that a significant clinical benefit was limited to patients identified as expressing only wild-type *KRAS* (21). In a phase III trial enrolling 572 advanced colorectal cancer patients that had failed previous chemotherapy, *KRAS* mutational status was again found to predict responsiveness to cetuximab therapy (22). Overall survival and progression-free survival in monotherapy-treated patients harboring *KRAS* wild-type tumors was approximately double that of patients receiving best supportive care, while patients with tumors harboring mutated KRAS did not significantly benefit from cetuximab therapy.

In the present studies, we have utilized these clinical findings to study the translational power of defined preclinical efficacy targets by evaluating the *KRAS* mutation status dependency of cetuximab benefits in 13 subcutaneous xenograft models of colorectal cancer. *EGFR* gene copy number and *BRAF* gene mutation status (23) were also evaluated as additional potential biomarkers predictive of EGFR antibody efficacy (24, 25).

Materials and Methods

Human cancer cell lines. Human colon carcinoma cell lines Colo320M, DLD-1, HCT-8, HCT-116, DLD-1, HT-29, LoVo, Ls174T, SW48, SW620, and T84 were obtained from the American Type Culture Collection (Manassas, VA, USA), and KM-12 cells were from the Division of Cancer Treatment and Diagnosis Tumor Repository of the National Cancer Institute (Frederick, MD, USA). DiFi cells were kindly provided by Dr. Zhen Fan and GEO cells by Dr. Lee Ellis. Cells were maintained at 37˚C in 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA, USA; Colo320DM, HCT-8, HCT-116, KM12), DMEM (Invitrogen; DLD-1, T84), McCoy's 5A (Invitrogen; GEO, HT-29), DMEM/F12 (Invitrogen; DiFi, LoVo) or MEM (Invitrogen; Ls174T) media supplemented with 10% defined fetal bovine serum (FBS; HyClone, Lenexa, KY, USA) and 2 mM GlutaMAX® (Invitrogen). Cells were routinely passaged utilizing TrypLE-Express (Invitrogen) treatment. Leibovitz's L-15 medium (Invitrogen) supplemented with FBS and GlutaMAX was utilized to maintain SW48 and SW620 cells, without CO_2.

Mice. Female athymic (*nu/nu*) mice, aged 7-8 weeks, were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice were housed under pathogen-free conditions in microisolator cages with laboratory chow and water available *ad libitum*. All experiments and procedures were approved by an Internal Animal Care and Use Committee and performed in accordance with the United States Department of Agriculture and the National Institute of Health policies regarding the humane care and use of laboratory animals.

Treatments. Cetuximab was produced by ImClone Systems (Somerville, NJ, USA), diluted in USP Saline (B. Braun Medical, Allentown, PA, USA) and dosed intraperitoneally on a Monday-Wednesday-Friday schedule at 1 mg/dose. Oxaliplatin (LKT Laboratories, St. Paul, MN, USA) was prepared in a solution of 5% USP dextrose. Irinotecan (LKT Laboratories) was prepared with 2.25 mg sorbitol per mg of irinotecan in 5% dextrose. Cytotoxic therapies were administered intraperitoneally on a *q3w* schedule (26). USP saline was dosed intraperitoneally Monday-Wednesday-Friday at 0.5 ml/dose as a control.

Irinotecan+Oxaliplatin (IROX) maximum tolerated dose (MTD). HT-29 colorectal cancer xenografts were established by injecting 5×10^6 tumor cells/mouse subcutaneously as described previously (27). Tumor volume was measured twice each week with calipers utilizing the formula: tumor volume=$(\pi/6\ (w_1\times w_2\times w_2))$, where w_1 represents the largest tumor diameter and w_2 represents the diameter perpendicular to w_1. Mice were randomized by tumor volume into treatment groups after the mean tumor volume reached approximately 250 mm^3. Oxaliplatin was dosed at 18, 12, and 6 mg/kg, followed by irinotecan at 200, 150, or 100

mg/kg. Survival, body weight and tumor volume were evaluated throughout the studies to guide the establishment of an MTD.

Subcutaneous colorectal cancer xenograft models. Colorectal cancer xenografts were established by injecting 5-10×10^6 tumor cells/mouse subcutaneously as described above. Mice were randomized by tumor volume into treatment groups after the mean tumor volume reached approximately 200-300 mm^3. Irinotecan (200 mg/kg) plus oxaliplatin (6 mg/kg) (IROX), irinotecan alone, and oxaliplatin alone were tested for combination effects with cetuximab. Chemotherapy dosing started one day prior to the start of cetuximab and saline dosing.

Statistical analysis. The treatment/control percentage (T/C%) was calculated as 100× ratio of the relative tumor volumes (RTV) in the experimental *versus* the control groups, with RTV=final mean tumor volume/initial mean tumor volume. Tumor growth was compared by repeated measures ANOVA using JMP Statistical software (v. 8.0; SAS Institute, Cary, NC, USA). A tumor was considered to have partially regressed if the final individual mouse tumor volume was reduced by at least 30% compared to the pre-treatment value. Regression frequency was compared by Chi-squared test. $P \leq 0.05$ was considered statistically significant.

DNA isolation. DNA was isolated from human cell lines by use of QiaAmp DNA Mini kits (Qiagen, Valencia, CA, USA) per the manufacturer's instructions.

KRAS sequencing. A polymerase-chain reaction (PCR) was performed to amplify exon 2 of the *KRAS* gene in DNA samples utilizing the following oligonucleotide primers: 5'-TAAGGCCTGCTGAAAA TGACTG-3' and 5'-TGGTCCTGCACCAGTAATATGC-3'. The reaction was run on a Px2 Thermal Cycler (Thermo Electron, Rockford, IL, USA) utilizing Platinum HiFi PCR Supermix (Invitrogen), 50-200 ng of genomic DNA, and a final concentration of 200 nM of each oligonucleotide, according to manufacturer's instructions. The reaction yielded a final product of 166 base pairs (bp), confirmed by electrophoresis on a 1.2% agarose, TAE gel. Bands from the PCR product electrophoresis were excised and purified using QIAquick Gel Extraction kits (Qiagen). The gel-purified PCR product was then sequenced on an ABi Prism 3700 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) utilizing the oligonucleotides that generated the product. Sequence data was analyzed utilizing Vector NTI software (Invitrogen).

Determination of mutations located on exon 3 of the *KRAS* gene in cell line DNA samples was carried out using the same methods above with the following oligonucleotides: 5'-GAGCAGGAAC AATGTCTTTTC-3' and 5'-CAGGGATATTACCTACCTC-3'. PCR performed as described above yielded a final PCR product of 449 bp. Attempts to sequence the 449 bp PCR product utilizing the same oligonucleotides from the amplification step were unsuccessful. The following exon 3-targeted primers were successfully utilized: 5'-CCTTTGCCCATTTTTAAATTG-3' and 5'-CTATAATTACTCCTTA ATGTC-3'. Chromatogram data were analyzed as above for the determination of mutation status.

BRAF sequencing. PCR was performed to amplify exon 15 of the *BRAF* gene utilizing the following oligonucleotide primers: 5'-GACATACTTATTGACTCTAAG-3' and 5'-GACCTTCAATGACTTT CTAG-3'. The polymerase chain reaction was set up using Platinum HiFi PCR Supermix, 50-200 ng of genomic DNA and a final concentration of 200 nM for each oligonucleotide. The PCR protocol described above was followed, yielding a 359 bp product. Bands from the PCR product electrophoresis were excised, purified, and sequenced as described, utilizing the oligonucleotides that generated the product. Sequence data was then analyzed with Vector NTI software to determine *BRAF* mutation status.

EGFR gene copy number. RT-PCR Copy Number Assay for the *EGFR* gene was run simultaneously with a TaqMan® Copy Number Reference Assay RNase P using RT-PCR Genotyping Master Mix (2×) in a duplex real-time PCR (Applied Biosystems). The RT-PCR Reference Assay detects a sequence of RNase-P H1 RNA as a control for a two copy gene. The gene copy number for *EGFR* and RNAse-P H1 in each test sample was determined using a 7500 Real-Time PCR System and CopyCaller™ Software (Applied Biosystems). The CopyCaller™ Software utilizes a comparative CT (ddCT) method that compares the dCT values of each cell line sample to normal human male DNA as a calibrator sample with a known number of *EGFR* gene copies.

Fluorescence in situ hybridization (FISH). FISH for *EGFR* and the chromosome 7 marker *CEP7* was performed on formalin-fixed paraffin-embedded DiFi and HT-29 xenograft tumor sections, established as described above. FISH analysis was performed utilizing the LSI *EGFR* Spectrum Orange/ CEP7 SpectrumGreen probe set according to the manufacturer's instructions (Vysis/Abbott Molecular, Des Plaines, IL, USA). Briefly, tumor tissue sections were deparaffinized in CitriSolc (Fisher Scientific, Pittsburgh, PA, USA) and dehydrated through 100% ethanol. Following proteinase K digestion, gene probes were applied. Specimens were then heated to denature unhybridized chromosomal and probe DNA. Following washing and dehydration, DAPI in anti-fade solution (Vector Laboratories, Burlingame, CA, USA) was pipetted onto the specimen for nuclear counterstaining. An epifluoresence microscope (Nikon Eclipse 90i) was utilized to visualize *EGFR* and *CEP7* gene signals.

EGFR immunohistochemistry (IHC). IHC on 5 μm formalin-fixed paraffin-embedded xenograft tumor sections was performed as described elsewhere (28).

Results

KRAS and BRAF mutation status. Potential genetic predictive biomarkers for EGFR antibody antitumor effects were evaluated for human colorectal cancer cell lines. Table I reports the results of sequencing of exon 2 (encompassing codons 12 and 13) and exon 3 (encompassing codon 61) in the *KRAS* gene, and exon 15 in the *BRAF* gene, from DNA samples isolated from 13 colorectal cancer cell lines. Six of the 13 cell lines harbored mutations in the *KRAS* gene affecting amino acids 12 or 13 in the KRAS protein (Table I). Additional *KRAS* mutations reported in colorectal cancer patients affecting amino acids 61 (29) and 146 (30) were not found in the tested cell lines. Two of the *KRAS* wild-type cell lines harbored a *BRAF* V600E mutation (Table I).

EGFR copy number. EGFR gene copy number is another potential biomarker for EGFR antibody response (31). By RT-PCR, *EGFR* gene copy number in colorectal cancer cell lines ranged from normal at 2 copies (Colo320DM and KM-12) to exceedingly high at 535 copies (DiFi) (Table I). In patient

Table I. *KRAS and BRAF mutational status for colorectal cancer cell lines utilized to establish xenograft tumors. Sequences were determined by PCR as described in the Materials and Methods. The KM12 cell line demonstrated a subpopulation of cells expressing the V600E mutation (1799T>A). EGFR gene copy number was evaluated utilizing an RT-PCR Copy Number Assay for the EGFR gene.*

Cell line	*KRAS*		*BRAF*		*EGFR* Copy #
Colo320DM	WT		WT		2
DiFi	WT		WT		535
Ls174T	WT		WT		7
SW48	WT		WT		4
T84	WT		WT		10
HT-29	WT		V600E	Heterozygous	10
KM12	WT		V600E	Subpolulation	2
GEO	G12A	Homozygous	WT		9
DLD-1	G13D	Heterozygous	WT		4
HCT-8	G13D	Heterozygous	WT		3
HCT-116	G13D	Heterozygous	WT		6
LoVo	G13D	Homozygous	WT		5
SW620	G13V	Homozygous	WT		9

samples, *EGFR* gene copy number is typically evaluated by counting the fluorescent dots per nucleus in sections stained by FISH. FISH analysis of patient tumors typically assigns >20 copies to tumor cells with very high *EGFR* copy number (32). FISH analysis of DiFi cells in subcutaneous xenograft tumors demonstrated a clustered pattern of *EGFR* gene staining (Figure 1A) similar to that reported in a subset of cells isolated from Japanese NSCLC patients (33). This pattern would be assigned a value of >20 copies with standard counting techniques but RT-PCR analysis showed the copy number to be much higher than 20. Since the signal for the chromosome 7 marker CEP7 did not show a clustered pattern in DiFi cells, these cells are considered to be an *EGFR* amplified tumor cell line. For comparison, FISH stained HT-29 cells in subcutaneous xenograft tumors generally exhibited corresponding CEP7 staining with each *EGFR* dot, indicating polysomy 7 (Figure 1B). In line with copy number results, immunohistochemical staining for EGFR found much higher EGFR immunoreactivity in DiFi cells than HT-29 cells in xenograft tumors (Figure 1C, D).

IROX MTD. Cetuximab increases the antitumor effects of oxaliplatin in preclinical models of colorectal cancer (34), and increases the effects of irinotecan in preclinical models (27) and colorectal cancer patients (35). IROX was therefore tested in combination with cetuximab in an effort to maximize antitumor efficacy. To determine the MTD of IROX, oxaliplatin and irinotecan were combined in a matrix of combination doses in the HT-29 subcutaneous xenograft tumor model. Oxaliplatin at 18 mg/kg, combined with 100, 150, or 200 mg/kg irinotecan, was toxic to mice and no animals survived the first cycle of *q3w* chemotherapy dosing (Figure 2A). With 12 mg/kg oxaliplatin, no mice survived to the second dose when combined with 200 mg/kg irinotecan, and significant weight loss was observed with 150 mg/kg irinotecan, although all animals in the latter group survived the entire dosing schedule. The combinations of 12 mg/kg oxaliplatin +100 mg/kg irinotecan and 6 mg/kg oxaliplatin +200 mg/kg irinotecan did not cause significant weight loss and all mice survived the study. In addition, these two regimens achieved similar inhibition of HT-29 xenograft tumor growth (Figure 2B). Based on this data, and the desire to limit the risk of toxicity in subsequent studies, oxaliplatin at 6 mg/kg + irinotecan at 200 mg/kg was selected as the MTD of this chemotherapeutic regimen to be utilized in combination studies with cetuximab as reported below.

Biomarker status versus antitumor efficacy. Figure 3 reports the efficacy (T/C% and partial regression frequency) of cetuximab, IROX, and cetuximab+IROX in a panel of subcutaneous colorectal cancer xenograft models, grouped by *KRAS* status. T/C% is a measure of relative tumor growth where T/C%=0% reflects complete tumor regression and T/C%=100% indicates no treatment effect. Cetuximab+IROX combination had a better effect than either treatment alone, according to both measures of efficacy. Given the previously noted clinical dependence of cetuximab therapeutic benefits on *KRAS* status, it is noteworthy that two *KRAS* wild-type models, Colo320 and Ls174T, were relatively resistant to cetuximab+IROX therapy (Figure 3), while three models harboring a mutant *KRAS* gene, GEO, HCT-8, and LoVo, had a T/C% of <20% with this combination (Figure 3A). The presence of a *BRAF* mutation in the HT-29 and KM-12 models (Table I) did not obviously impact efficacy compared to other *KRAS* wild-type models harboring only wild-type *BRAF* (Figure 3). Very high *EGFR* gene copy number, found only in the DiFi model, appeared to hold some predictive potential as this model was especially responsive to cetuximab.

T/C% <50% as a target. A T/C% of <50% has been utilized as a threshold in preclinical subcutaneous xenograft tumor models for claiming meaningful treatment activity. Across the panel of 13 models tested, T/C% <50% (Figure 3A) was achieved with cetuximab monotherapy in 57% of *KRAS* wild-type models and 50% of *KRAS* mutant models. For cetuximab+IROX this level of efficacy was achieved in all *KRAS* mutant models and 71% of *KRAS* wild-type models. Clearly had the present data been available prior to patient testing, a target of T/C%≤50% would not have predicted the now established difference in the clinical benefits of cetuximab between patients harboring *KRAS* mutant and wild-type tumors.

Tumor regression as a target. Figure 3B shows the effect of cetuximab, IROX, and cetuximab+IROX on the incidence of partial tumor regressions. The only model in which cetuximab monotherapy significantly increased the frequency of tumor regressions was the *KRAS* wild-type DiFi model (Figures 3B and

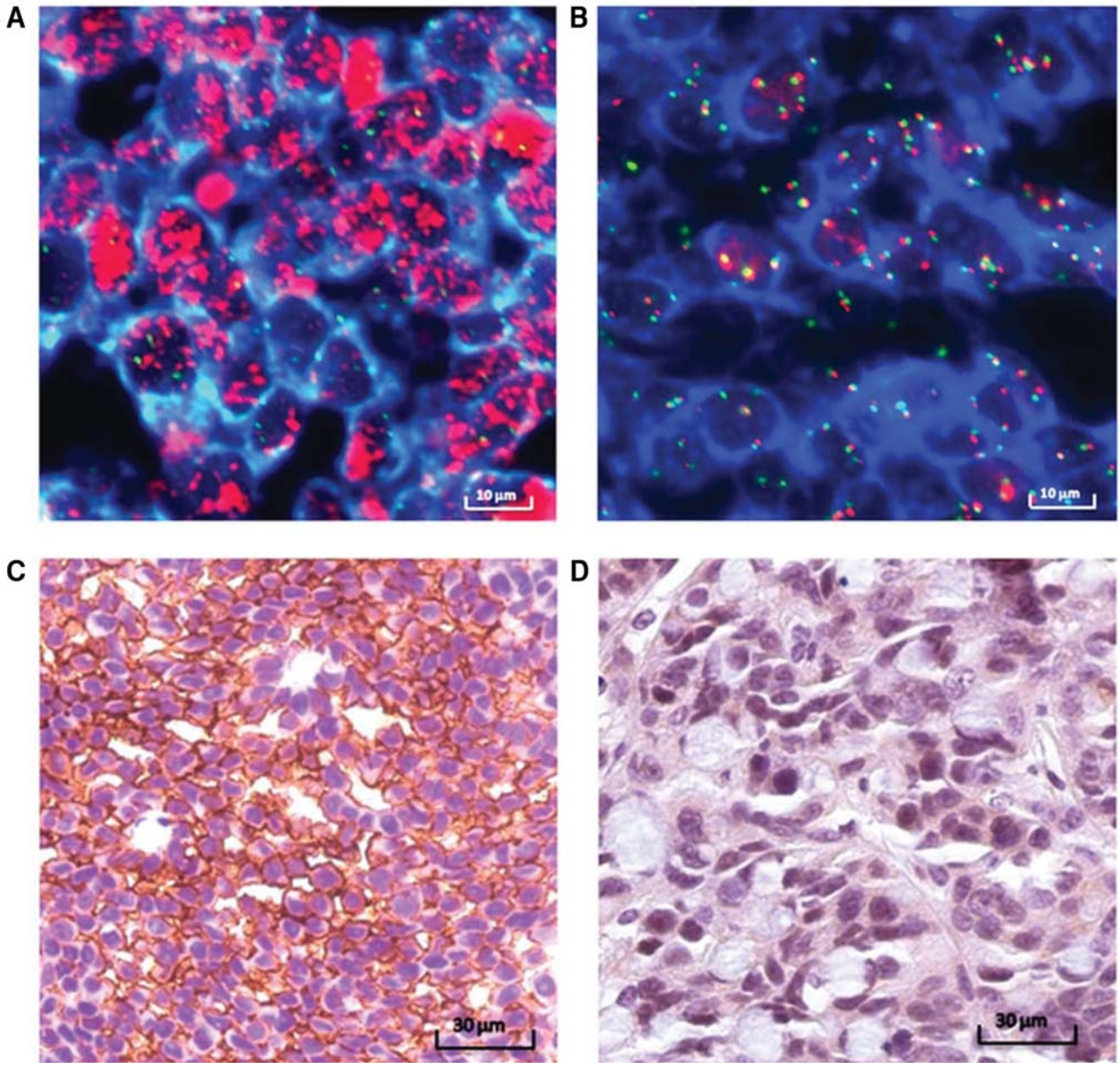

Figure 1. *Fluorescence in situ hybridization for EGFR copy number in DiFi (A) and HT-29 (B) tumors. EGFR (red), CEP7 (green), ×1200. Immunohistochemistry for EGFR in DiFi (C) and HT-29 (D) tumors.*

4). When cetuximab was given in combination with IROX, the percentage of models with greater than 10% of tumors regressing was 50% for *KRAS* mutant tumors and 43% for *KRAS* wild-type tumors (Figure 3B).

Source of IROX combination benefits. Cetuximab+IROX consistently achieved greater antitumor effects than either treatment alone. To understand the treatment requirements for this benefit, we performed studies to determine whether both cytotoxic therapies were required to achieve a maximal effect, or if one of the cytotoxic therapies could be omitted. In the 9 tumor models in which statistically significant combination benefits of cetuximab+IROX were detected, a second study was performed in which cetuximab was combined with irinotecan, oxaliplatin, or IROX (Table II, Figure 5). The two-drug combination of cetuximab+irinotecan consistently achieved similar efficacy to that of cetuximab+IROX ($p>0.05$ by RM ANOVA for all), unlike cetuximab+oxaliplatin. This indicates that the contributions of irinotecan significantly exceeded that of oxaliplatin. However, in spite of the lack of statistical

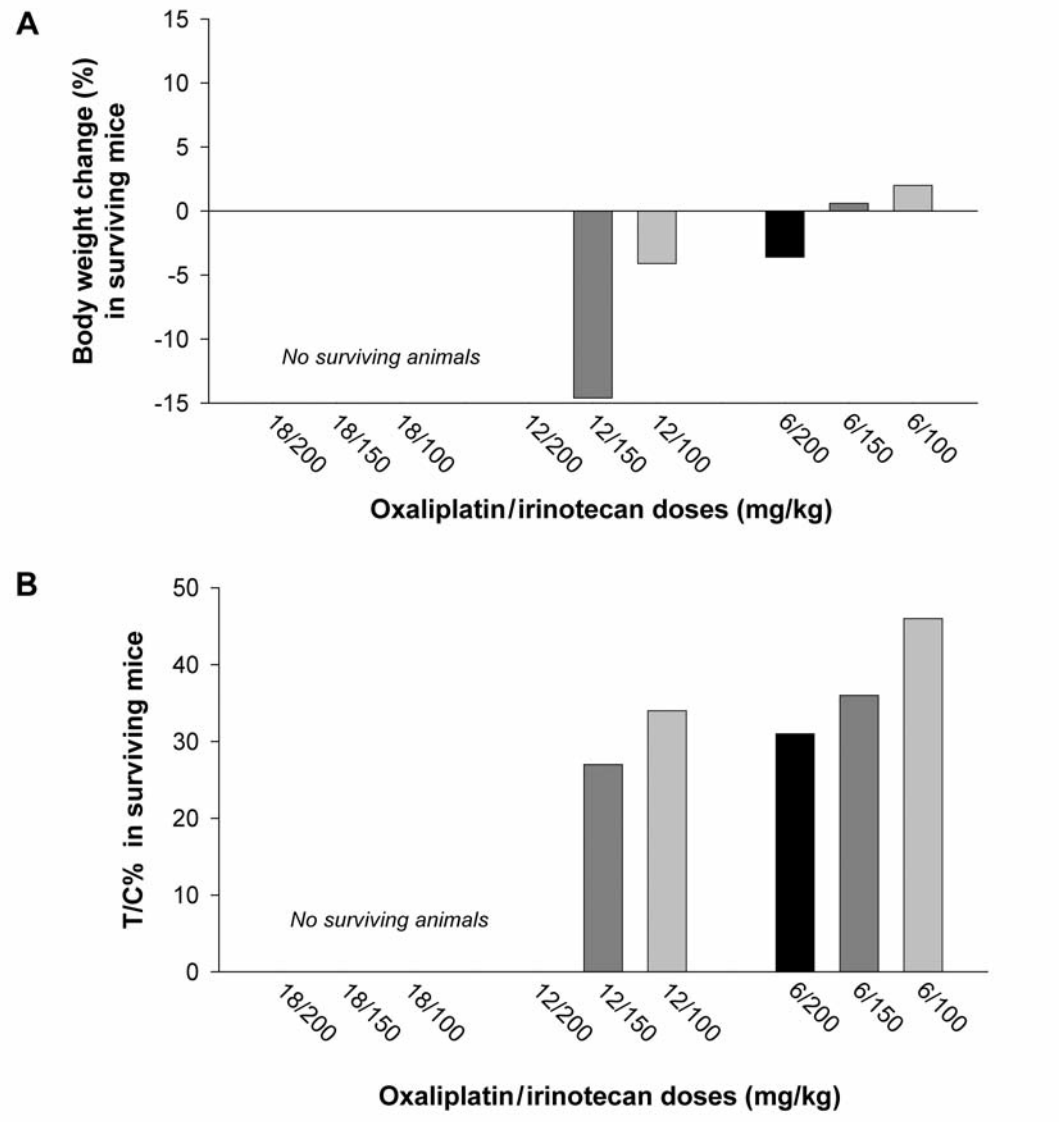

Figure 2. *Response of HT-29 xenografts to oxaliplatin/irinotecan combination therapy. A: Percentage change in body weight and B: T/C %, calculated as 100×ratio of the relative tumor volumes (RTV) in the experimental versus the control groups, where RTV=final mean tumor volume/initial mean tumor volume.*

significance, whether considering the T/C% values (Figure 5A) or the partial tumor regression frequency (Figure 5B), cetuximab+IROX tended to have greater efficacy than cetuximab+irinotecan in all 9 models evaluated.

Discussion

The decision to advance a novel cancer therapeutic into clinical testing initiates the investment of significant resources. These resources are both monetary and human, and include the time of cancer patients hopeful that an experimental therapy will extend their life. It is therefore important to rationally determine the criteria to be utilized for prioritizing novel treatment strategies for clinical testing. In the present research, two targets for preclinical efficacy in subcutaneous tumors models established in mice were considered: (i) T/C% <50% and (ii) greater than 30% reduction in tumor volume in >10% of treated mice. As a measure of the utility of these criteria, we determined their capacity to predict for a differential benefit of cetuximab in colorectal cancer models harboring mutant or only wild-type *KRAS*. Unexpectedly, targeting the ability to inhibit subcutaneous xenograft tumor growth with T/C%<50% did not find *KRAS* mutation status to be a response biomarker, as has been demonstrated in colorectal cancer patients treated with EGFR-targeted monoclonal antibodies. Tumor regression with cetuximab monotherapy on the other hand, although infrequent,

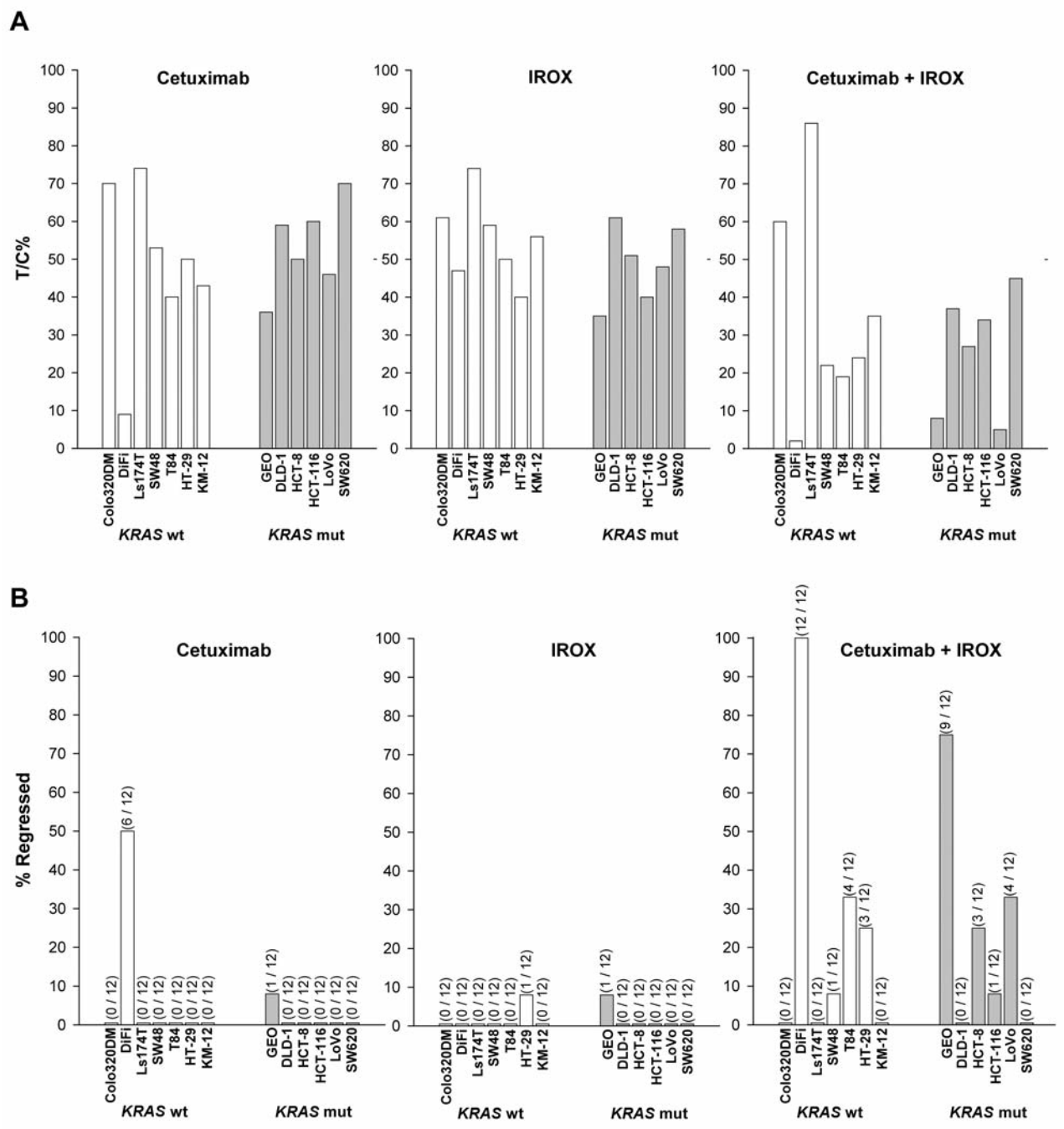

Figure 3. *Response of 13 subcutaneous colon carcinoma xenograft models to treatment with cetuximab, irinotecan+oxaliplatin (IROX), and cetuximab+IROX. A: T/C% and B: percentage of animals per group with partial tumor regressions (>30% reduction in tumor volume compared to pretreatment tumor volume) in models established with the indicated human colorectal cancer cell lines. Treatment duration ranged from 15 to 46 days, guided by the aggressiveness of each model (n=12 mice per treatment group). Cells are grouped into KRAS wild-type (wt) and mutant (mut), as detailed in Table I.*

was only found in a model harboring wild-type *KRAS*. These data support the idea that requiring more dramatic responses than just tumor growth inhibition in preclinical models may lead to conclusions that better translate into the clinic.

Standards utilized for a target level of preclinical efficacy in subcutaneous tumor models have generally been in the range of T/C% ≤ 42-50% (6 and 7), although statistically significant (p<0.05) tumor growth inhibition at T/C% ≥ 50% has also been

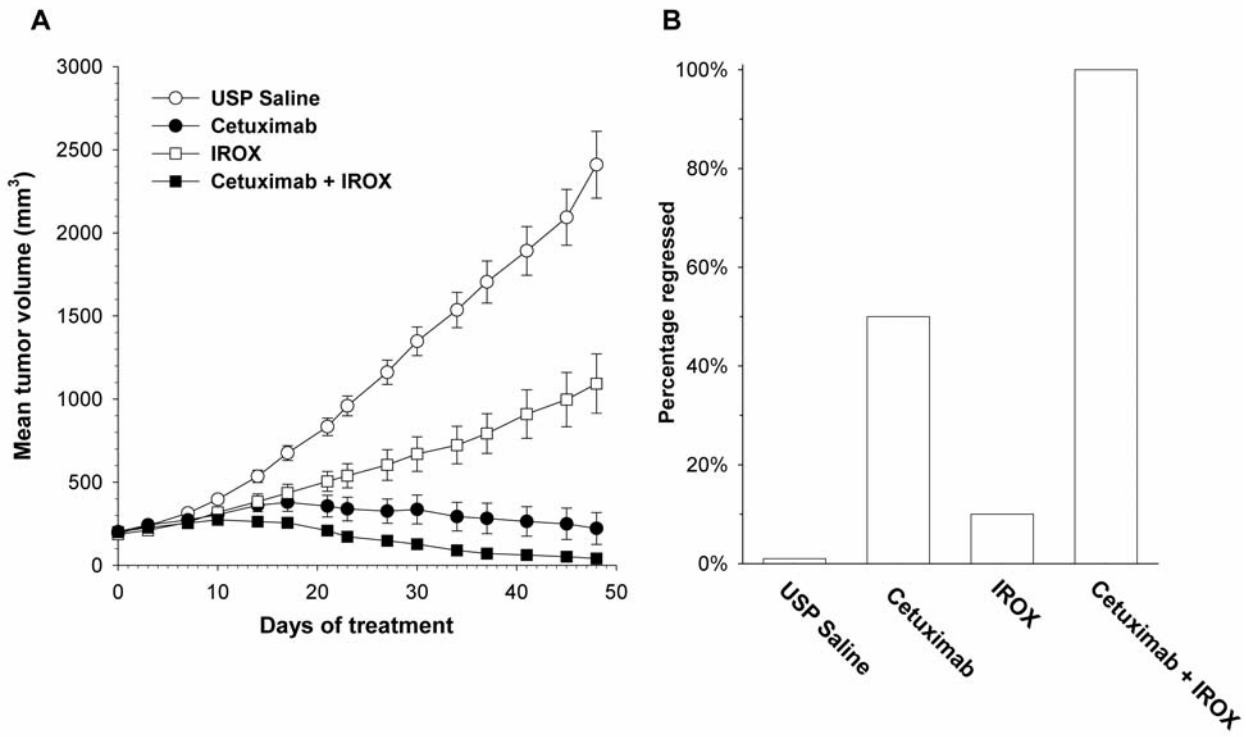

Figure 4. *Effect of cetuximab+IROX in the DiFi human xenograft model. A: Effect of treatment on the growth of DiFi xenograft tumors and B: percentage of animals per group with tumor regressions. Regression was defined as a 30% reduction in tumor volume at the end of treatment compared to day 0, where the formula end tumor volume–(day 0 tumor volume ×0.7) yielded a negative number. Bars, ±S.E.M.*

Table II. *Efficacy of the indicated treatments as measured by T/C% and the number of animals per treatment group with partial tumor regressions (number regressed animals/total number of animals in the treatment group).*

	Cetuximab + Oxaliplatin		Cetuximab + Irinotecan		Cetuximab + IROX	
Tumor model	T/C%	Partial regressions	T/C%	Partial regressions	T/C%	Partial regressions
KRAS WT						
SW48	60%	0/12	17%	3/12	12%	5/12
T84	46%	3/12	22%	5/12	19%	7/12
HT-29	62%	0/12	47%	0/12	24%	0/12
KM-12	69%	0/12	49%	0/12	35%	0/12
KRAS MUT						
DLD-1	66%	0/12	50%	0/12	37%	0/12
GEO	20%	1/12	13%	5/12	8%	9/12
HCT-8	51%	0/12	39%	0/12	27%	2/12
HCT-116	90%	0/12	41%	0/11	34%	1/12
LoVo	28%	2/12	14%	6/12	5%	7/11

reported as a positive result (*e.g.* 36 and 37). In the clinic, continued tumor growth is considered progressive disease (8), so the rationale for advancing drugs under standards such as T/C% <50% without requiring tumor regression is likely related to the belief that when hundreds of patients are tested in clinical trials, individual patients may have objective responses (tumor regression) to treatment, even if responses were not observed in animal models of cancer. In other words, tumor growth-inhibitory effects detected in animal models may be predictive for objective responses in some patients, utilizing Response Evaluation Criteria in Solid Tumors (RECIST) (38) criteria to define response (*e.g.* 30% decrease in the sum of longest

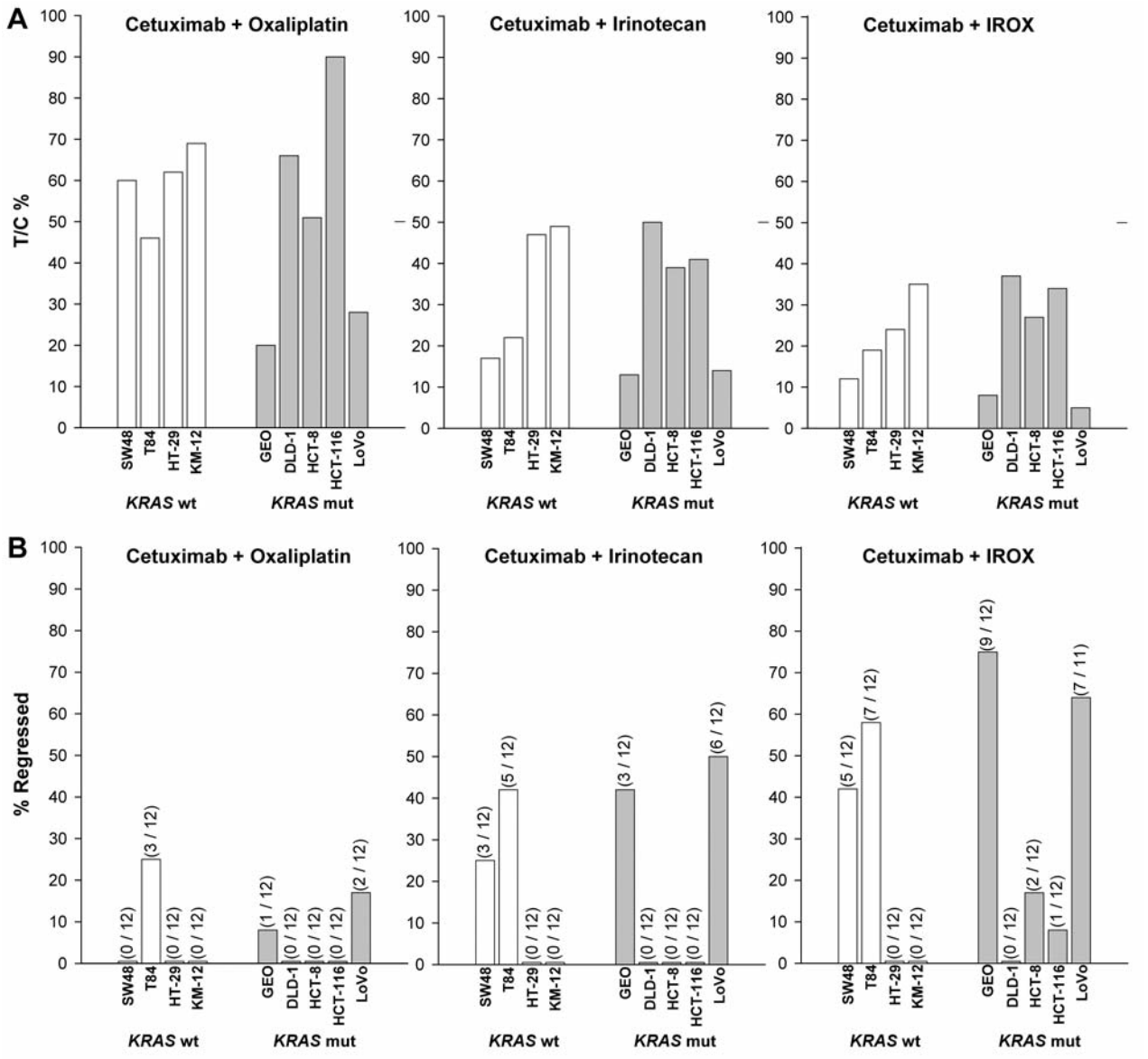

Figure 5. *Tumor response to treatment with cetuximab+IROX, cetuximab+oxaliplatin, and cetuximab+irinotecan. A: T/C% and B: percentage of animals per group with partial tumor regressions (>30% reduction in tumor volume compared to pretreatment tumor volume) in models established with the indicated human colorectal cancer cell lines. Models selected were those that showed a significant combination benefit of cetuximab+IROX in studies reported in Figure 3. Treatment duration ranged from 28 to 60 days, guided by the aggressiveness of each model (n=12 mice per treatment group). Cells are grouped into KRAS wild-type (wt) and mutant (mut), as detailed in Table I.*

diameters of target lesions). One clear method to evaluate this rationale is to compare well-accepted treatment-related findings reported in patients to the effects of treatment in cancer models.

Recently it has been demonstrated that the EGFR antibodies cetuximab (21 and 22) and panitumumab (39) have a clinically meaningful benefit in colorectal cancer patients with tumors harboring wild-type but not mutant *KRAS* (also see above). In recognition of this, the American Society for Clinical Oncology in 2009 issued a Provisional Clinical Opinion that patients with colorectal cancer who are candidates for EGFR antibody therapy should be tested for *KRAS* mutations, and those with mutations in codons 12 or 13 should not receive this therapy (40). However a somewhat more complicated story is emerging, indicating that the impact of *KRAS* mutation on cetuximab efficacy may differ depending on the specific mutation. In particular, a retrospective study found that patients with the G13D mutation had longer overall survival in response to cetuximab than patients with other *KRAS* mutations (41). This result was not consistent with a lack of preclinical efficacy reported for cetuximab in an early passage colon tumor xenograft model established with cells harboring a

G13D *KRAS* mutation (37), although the weekly doses of cetuximab and irinotecan utilized in this preclinical model were 12- and 27-fold less, respectively, than that utilized in the present research that focused on maximum achievable effects. Nevertheless, while the impact of *KRAS* mutation on cetuximab efficacy may differ depending on the specific mutation, the clinical findings indicating that a majority of patients with *KRAS* mutations in codons 12 or 13 fail to have clinically relevant benefits in response to cetuximab provide a means to evaluate proposed thresholds for preclinical activity.

When an efficacy standard of T/C%<50% was tested in 13 preclinical models of colorectal cancer, treatment with cetuximab or cetuximab+IROX inhibited tumor growth in *KRAS* mutant and wild-type models with very similar frequency. Therefore utilization of this target for preclinical activity would not have predicted for the dramatic difference in the efficacy of EGFR antibodies established in patients. It should be noted that this result is derived from the comparison of patient data with preclinical data, with regard to one example; response in *KRAS* mutant *versus* wild-type subcutaneous xenograft colorectal cancer models. Additional comparisons will therefore be necessary to strengthen this conclusion. However, when the aim is to utilize preclinical data to predict for treatment benefit in the clinic, our findings clearly suggest that inhibition of tumor growth without tumor regression in preclinical models should be considered progressive disease as it is in patients, rather than an example of drug activity supporting clinical development.

Tumor regression with cetuximab monotherapy in our models aligned with the clinical findings, namely that cetuximab only induced a significant frequency of tumor regressions in a *KRAS* wild-type model, DiFi. Tumor regression indicates significant tumor cell death, often by apoptosis in response to cell damage caused, for example, by DNA-damaging chemotherapies. The cell death program may in addition be initiated by blocking the molecular pathways keeping pre-existing cellular damage or abnormalities from having this effect.

Tumor cell death in response to the targeting of a single molecular pathway is evidence of a pathway addiction under which some cancer cells survive and proliferate (23, 42). DiFi is an example of a cell line exhibiting an addiction to the EGFR pathway, accomplished through the 250-fold amplification of the *EGFR* gene, resulting in very high expression of this growth factor receptor (43). Amplification of the *EGFR* gene in colorectal cancer patients has been associated with clinical benefit for panitumumab and cetuximab (23, 24, 44), supporting the potential relevance of the findings with the DiFi model. Notably in the present work, tumors established with colorectal cancer cell lines exhibiting up to a 5-fold increase in *EGFR* gene copy number compared to normal were not addicted to the EGFR pathway, given the lack of significant regressions with cetuximab therapy. This suggests that very high copy number, much greater than 10, will be predictive of response to EGFR antibody therapy.

Tumor regression with cetuximab+IROX, as with T/C%<50%, did not predict for *KRAS* mutation as a response biomarker for this combination therapy, in spite of the fact that cetuximab was required to achieve significant regressions. The efficacy of cetuximab+IROX in preclinical models was mostly due to the combination of cetuximab+irinotecan. In the clinic, the benefits of cetuximab+irinotecan have also been reported to be dependent on *KRAS* mutation status (25) so the finding of similar frequencies of *KRAS* mutant and wild-type models with significant tumor regressions indicates this criterion is lacking in its ability to generate conclusions that translate into the clinic for strategies combining a targeted agent with cytotoxic therapy.

Xenografts established with the GEO cell line, which is homozygous for the G12A *KRAS* mutation, had significant regression with cetuximab+IROX therapy. The responsiveness of this cell line to EGFR ligand, despite a mutated constitutively active *KRAS*, may be due to signaling through other RAS isoforms (45). Thus this cell line may not be representative of the typical *KRAS* mutant cancer cell that contributes towards disease progression and reduced survival in patients. Likewise, the G13D mutation expressed in the SW48 colorectal cancer cell line does not obviate the need for EGFR activity in support of tumor progression (41). These data suggest that *KRAS* mutation in certain cellular environments may not always predict for a lack of benefit of EGFR antibodies. In support of this, although not satisfying RECIST criteria for response, stable disease was noted as a response to cetuximab monotherapy in 3 out of 30 patients with *KRAS* mutant colorectal cancer (46). Others have even reported rare objective responses with cetuximab alone or in combination with chemotherapy in patients with *KRAS* mutant colorectal cancer (41).

In fact the ability of cetuximab to reduce tumor growth and increase the effects of chemotherapy in most colorectal cancer models utilized in the present study, independent of *KRAS* status, may indicate that tumor growth can be slowed in patiens with *KRAS* mutant by EGFR antibody therapy, even though an important clinical benefit on survival or tumor regression is not achieved. This impact of EGFR in cancer cells harboring mutant *KRAS* may be related to signaling downstream to EGFR, outside the KRAS pathway (47 and 48). Given the potential for an impact of EGFR antibodies in *KRAS* mutant colorectal cancer that fall below the threshold of RECIST criteria, it is important to note the continued decrease in T/C% and increase in regression frequency when adding oxaliplatin to cetuximab+irinotecan, in every model tested. Results such as these leave open the possibility that although EGFR antibodies are not currently achieving meaningful clinical benefits with current treatment regimens in *KRAS* mutant colorectal cancer, agents targeting EGFR may still be of benefit in future combination strategies for *KRAS* mutant colorectal cancer.

In conclusion, models for human disease are utilized because it is not feasible to test the existing volume of novel treatment strategies in patients. Yet clinical testing is in the end required to

establish meaningful benefits, making prioritization a major function of testing in models. In cancer research, the subcutaneous xenograft tumor model established with tumor cell lines is the workhorse of many laboratories, due to the volume of models available, the efficiency of testing many treatment strategies, and the ease of assessment of treatment efficacy. Here we have utilized clinical findings to evaluate criteria for treatment efficacy to be utilized to claim that a treatment 'works' in such models and should be advanced to clinical testing. Our results indicate that criteria should be elevated from current standards, in that major tumor responses, including regression, should be sought. In particular, for targeted therapies, models should be screened for examples of tumor cells addicted to the pathway(s) being targeted to support testing in patients.

References

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: GLOBOCAN 2008, Cancer incidence and mortality worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010 (available from: http://globocan.iarc.fr).

2 Adams CP and Brantner VV: Estimating the cost of new drug development: Is it really $802 million? Health Affairs *25*: 420-428, 2006.

3 Reichert JM and Wenger JB: Development trends for new cancer therapeutics and vaccines. Drug Discov Today *13*: 30-37, 2008.

4 Hollingshead MG: Antitumor efficacy testing in rodents. J Natl Cancer Inst *100*: 1500-1510, 2008.

5 Boven E, Winograd B, Berger DP, Dumont MP, Braakhuis BJ, Fodstad O, Langdon S and Fiebig HH: Phase II preclinical drug screening in human tumor xenografts: a first European multicenter collaborative study. Cancer Res *52*: 5940-5947, 1992.

6 Alley MC, Hollingshead MG, Dykes DJ and Waud WR: Human tumor xenograft models in NCI drug development. *In*: Cancer Drug Discovery and Development: Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval. Teicher BA and Andrews PA (eds.). Totowa, NJ., Humana Press Inc., pp. 125-152, 2004.

7 Corbett T, Polin L, LoRusso P, Valeriote F, Panchapor C, Pugh S, White K, Knigh J, Demchik L, Jones J, Jones L and Lisow L: *In vivo* methods for screening and preclinical testing: Use of rodent solid tumors for drug discovery. *In*: Cancer Drug Discovery and Development: Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval. Teicher BA and Andrews PA (eds.). Totowa, NJ, Humana Press Inc., pp. 99-123, 2004.

8 Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D and Verweij J: New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer *45*: 228-247, 2009.

9 Bruns CJ, Liu W, Davis DW, Shaheen RM, McConkey DJ, Wilson MR, Bucana CD, Hicklin DJ and Ellis LM: Vascular endothelial growth factor is an *in vivo* survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer *89*: 488-499, 2000.

10 Philip PA, Benedetti J, Corless CL, Wong R, O'Reilly EM, Flynn PJ, Rowland KM, Atkins JN, Mirtsching BC, Rivkin SE, Khorana AA, Goldman B, Fenoglio-Preiser CM, Abbruzzese JL, and Blanke CD: Phase III study comparing gemcitabine plus cetuximab *versus* gemcitabine in patients with advanced pancreatic adenocarcinoma: Southwest Oncology Group-directed intergroup trial S0205. J Clin Oncol *28*: 3605-3610, 2010.

11 Okines A and Cunningham D: Current perspective: bevacizumab in colorectal cancer – A time for reappraisal? Eur J Cancer *45*: 2452-2461, 2009.

12 Rolland D, Camara-Clayette V, Barbarat A, Salles G, Coiffier B, Ribrag V and Thieblemont C: Farnesyltransferase inhibitor R115777 inhibits cell growth and induces apoptosis in mantle cell lymphoma. Cancer Chemother Pharmacol *61*: 855-863, 2008.

13 Rolland D, Ribrag V, Haioun C, Ghesquieres H, Jardin F, Bouabdallah R, Franchi P, Briere J, De Kerviler E, Chassagne-Clement C, Raponi M, Houlgatte R, Jais JP and Thieblemont C: Phase II trial and prediction of response of single-agent tipifarnib in patients with relapsed/refractory mantle cell lymphoma: a Groupe d'Etude des Lymphomes de l'Adulte trial. Cancer Chemother Pharmacol *65*: 781-790, 2010.

14 Chua W, Kho PS, Moore MM, Charles KA, and Clarke SJ: Clinical, laboratory and molecular factors predicting chemotherapy efficacy and toxicity in colorectal cancer. Crit Rev Oncol Hematol in Press, 2011

15 Richmond A and Su Y: Mouse xenograft models vs. GEM models for human cancer therapeutics. Disease Model Mech *1*: 78-82, 2008.

16 Saridaki Z, Georgoulias V and Souglakos J: Mechanisms of resistance to anti-EGFR monoclonal antibody treatment in metastatic colorectal cancer. World J Gastroenterol *16*: 1177-1187, 2010.

17 Boguski MS and McCormick F: Proteins regulating Ras and its relatives. Nature *366*: 643-654, 1993.

18 Shaw RJ and Cantley LC: Ras, PI(3)K and mTOR signaling controls tumour cell growth. Nature *441*: 424-430, 2006.

19 Schubbert S, Shannon K and Bollag G: Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer *7*: 295-308, 2007.

20 Siddiqui AD and Piperdi B: *KRAS* mutation in colon cancer: A marker of resistance to EGFR-I therapy. Ann Surg Oncol *17*: 1168-1176, 2010.

21 Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F and Laurent-Puig P: *KRAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res *66*: 3992-3995, 2006.

22 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ and Zalcberg JR: *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med *17*: 1757-1765, 2008.

23 Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S and Bardelli A: Gene copy number for epidermal growth factor receptor (*EGFR*) and clinical response to anti-EGFR treatment in colorectal cancer: a cohort study. Lancet Oncol *6*: 279-286, 2005.

24 Lièvre A, Rouleau E, Buecher B and Mitry E: Clinical significance of *BRAF* mutations in colorectal cancer. Bull Cancer *97*: 1441-1452, 2010.

25 Fornaro L, Baldi GG, Masi G, Allegrini G, Loupakis F, Vasile E, Cupini S, Stasi I, Salvatore L, Cremolini C, Vincenzi B, Santini D, Tonini G, Graziano F, Ruzzo A, Canestrari E, Magnani M and Falcone A: Cetuximab plus irinotecan after irinotecan failure in

elderly metastatic colorectal cancer patients: Clinical outcome according to *KRAS* and *BRAF* mutational status. Crit Rev Oncol Hematol in Press, 2011.

26 Hoff PM, Wolff RA, Xiong H, Jones D, Lin E, Eng C, Dutta A, Bogaard KR and Abbruzzese JL: Phase II trial of combined irinotecan and oxaliplatin given every 3 weeks to patients with metastatic colorectal cancer. Cancer *106*: 2241-2246, 2006.

27 Prewett MC, Hooper AT, Bassi R, Ellis, LM, Waksal, HW, Hicklin, DJ: Receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. Clin Cancer Res *8*: 994-1003, 2002.

28 Patel D, Bassi R, Hooper A, Prewett M, Hicklin DJ and Kang X: Anti-epidermal growth factor receptor monoclonal antibody cetuximab inhibits EGFR/HER-2 heterodimerization and activation. Int J Oncol *34*: 25-32, 2009.

29 Oliveira C, Westra JL, Arango D, Ollikainen M, Domingo E, Ferreira A, Velho S, Niessen R, Lagerstedt K, Alhopuro P, Laiho P, Veiga I, Teixeira MR, Ligtenberg M, Kleibeuker JH, Sijmons RH, Plukker JT, Imai K, Lage P, Hamelin R, Albuquerque C, Schwartz S Jr, Lindblom A, Peltomaki P, Yamamoto H, Aaltonen LA, Seruca R and Hofstra RM: Distinct patterns of *KRAS* mutations in colorectal carcinomas according to germline mismatch repair defects and *hMLH1* methylation status. Hum Mol Genet *13*: 2303-2311, 2004.

30 Edkins S, O'Meara S, Parker A, Stevens C, Reis M, Jones S, Greenman C, Davies H, Dalgliesh G, Forbes S, Hunter C, Smith R, Stephens P, Goldstraw P, Nicholson A, Chan TL, Velculescu VE, Yuen ST, Leung SY, Stratton MR and Futreal PA: Recurrent *KRAS* codon 146 mutations in human colorectal cancer. Cancer Biol Ther *5*: 928-932, 2006.

31 Campanella C, Mottolese M, Cianciulli A, Torsello A, Merola R, Sperduti I, Melucci E, Conti S, Diodoro MG, Zeuli M, Paoletti G, Cognetti F and Garufi C: Epidermal growth factor receptor gene copy number in 101 advanced colorectal cancer patients treated with chemotherapy plus cetuximab. J Transl Med *8*: 36, 2010.

32 Jolly C, Michelland S, Rocchi M, Robert-Nicoud M and Vourc'h C: Analysis of the transcriptional activity of amplified genes in tumour cells by fluorescence *in situ* hybridization. Hum Genet *101*: 81-87, 1997.

33 Sasaki H, Shimizu S, Okuda K, Kawano O, Yukiue H, Yano M and Fujii Y: Epidermal growth factor receptor gene amplification in surgically resected Japanese lung cancer. Lung Cancer *64*: 295-300, 2009.

34 Prewett M, Deevi DS, Bassi R, Fan F, Ellis LM, Hicklin DJ and Tonra JR: Tumors established with cell lines selected for oxaliplatin resistance respond to oxaliplatin if combined with cetuximab. Clin Cancer Res *13*: 7432-7440, 2007.

35 Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I and Van Cutsem E: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med *351*: 337-345, 2004.

36 van der Horst EH, Chinn L, Wang M, Velilla T, Tran H, Madrona Y, Lam A, Ji M, Hoey TC and Sato AK: Discovery of fully human anti-MET monoclonal antibodies with antitumor activity against colon cancer tumor models *in vivo*. Neoplasia *11*: 355-364, 2009.

37 Fischer M, Yen W-C, Kapoun AM, Wang M, O'Young G, Lewicki J, Gurney A, and Hoey T: Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic *KRAS* mutations. Cancer Res *71*: 1520-1525, 2011.

38 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst *92*: 205-216, 2000.

39 Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD and Chang DD: Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol *26*: 1626-1634, 2008.

40 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF and Schilsky RL: American Society of Clinical Oncology provisional clinical opinion: testing for *KRAS* gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol *27*: 2091-2096, 2009.

41 De Roock W, Jonker DJ, Di Nicolantonio F, Tu D, Siena S, Arena S, Frattini M, Piessevaux H, Van Custum E, O'Callaghan CJ, Khambata-Ford S, Zalchberg JR, Karapetis CS, Bardelli A and Tejpar S: Association of *KRAS* p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. JAMA *304*: 1812-1820, 2010.

42 Felsher DW: Reversing cancer from inside and out: oncogene addiction, cellular senescence, and the angiogenic switch. Lymphat Res Biol *6*: 149-154, 2008.

43 Dolf G, Meyn RE, Curley D, Prather N, Story MD, Boman BM, Siciliano MJ and Hewitt RR: Extrachromosomal amplification of the epidermal growth factor receptor gene in a human colon carcinoma cell line. Genes Chromosomes Cancer *3*: 48-54, 1991.

44 Sartore-Bianchi A, Moroni M, Veronese S, Carnaghi C, Bajetta E, Luppi G, Sobrero A, Barone C, Cascinu S, Colucci G, Cortesi E, Nichelatti M, Gambacorta M and Siena S: Epidermal growth factor receptor gene copy number and clinical outcome of metastatic colorectal cancer treated with panitumumab. J Clin Oncol *25*: 3238-3245, 2007.

45 Buard A, Zipfel PA, Frey RS and Mulder KM: Maintenance of growth factor signaling through Ras in human colon carcinoma cells containing *K-ras* mutations. Int J Cancer *67*: 539-546, 1996.

46 Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, Wong TW, Huang X, Takimoto CH, Godwin AK, Tan BR, Krishnamurthi SS, Burris HA 3rd, Poplin EA, Hidalgo M, Baselga J, Clark EA and Mauro DJ: Expression of epiregulin and amphiregulin and *K-ras* mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol *25*: 3230-3237, 2007.

47 Guruswamy S, Rao CV: Multi-target approaches in colon cancer chemoprevention based on systems biology of tumor cell-signaling. Gene Regul Syst Bio *2*: 163-176, 2008.

48 Yamatodani T, Ekblad L, Kjellén E, Johnsson A, Mineta H and Wennerberg J: Epidermal growth factor receptor status and persistent activation of Akt and p44/42 MAPK pathways correlate with the effect of cetuximab in head and neck and colon cancer cell lines. J Cancer Res Clin Oncol *135*: 395-402, 2009.

Received March 21, 2011
Revised May 6, 2011
Accepted March 9, 2011