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ORIGINAL REPORT

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Molecular Response to Cetuximab and Efficacy of Preoperative Cetuximab-Based Chemoradiation in Rectal Cancer

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ABSTRACT

Purpose
To characterize the molecular pathways activated or inhibited by cetuximab when combined with chemoradiotherapy (CRT) in rectal cancer and to identify molecular profiles and biomarkers that might improve patient selection for such treatments.

Patients and Methods
Forty-one patients with rectal cancer (T3-4 and/or N+) received preoperative radiotherapy (1.8 Gy, 5 days/wk, 45 Gy) in combination with capecitabine and cetuximab (400 mg/m^2 as initial dose 1 week before CRT followed by 250 mg/m^2/wk for 5 weeks). Biopsies and plasma samples were taken before treatment, after cetuximab but before CRT, and at the time of surgery. Proteomics and microarrays were used to monitor the molecular response to cetuximab and to identify profiles and biomarkers to predict treatment efficacy.

Results
Cetuximab on its own downregulated genes involved in proliferation and invasion and upregulated inflammatory gene expression, with 16 genes being significantly influenced in microarray analysis. The decrease in proliferation was confirmed by immunohistochemistry for Ki67 ($P = .01$) and was accompanied by an increase in transforming growth factor-α in plasma samples ($P < .001$). Disease-free survival (DFS) was better in patients if epidermal growth factor receptor expression was upregulated in the tumor after the initial cetuximab dose ($P = .02$) and when fibro-inflammatory changes were present in the surgical specimen ($P = .03$). Microarray and proteomic profiles were predictive of DFS.

Conclusion
Our study showed that a single dose of cetuximab has a significant impact on the expression of genes involved in tumor proliferation and inflammation. We identified potential biomarkers that might predict response to cetuximab-based CRT.

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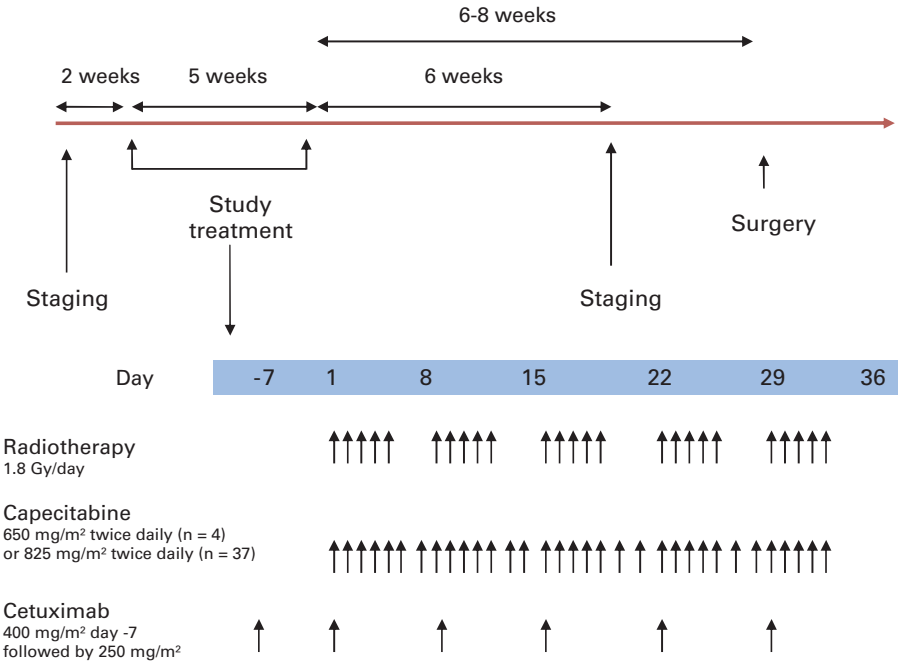
INTRODUCTION

Rectal cancer has a high risk of locoregional relapse that can cause significant morbidity and treatment failure. Preoperative chemoradiation (CRT) followed by total mesorectal excision (TME) is considered a standard treatment for stage II and III rectal cancer, decreasing the local relapse rate and improving clinical outcome.[1-5] Nevertheless, the risk of local relapse in this patient group remains approximately 8%. To further improve these results, targeted therapies that might selectively radiosensitize tumors are now being investigated.

Cetuximab (Erbitux, Merck, Darmstadt, Germany) is a chimeric immunoglobulin (Ig) G1 monoclonal antibody directed against the epidermal growth factor receptor (EGFR). EGFR is a member of the HER tyrosine kinase growth factor receptor family that signals cellular differentiation, proliferation, and survival. Cetuximab has demonstrated significant clinical activity in metastatic colorectal cancer.[6-8] In addition, cetuximab in combination with curative-intent radiotherapy has been reported to increase median survival over radiation alone in locally advanced head and neck carcinoma.[9]

We postulated that the addition of cetuximab to a preoperative concurrent radiotherapy and capecitabine regimen in patients with rectal cancer would improve pathologic response and clinical outcome.[10] Surprisingly, the pathologic complete response (pCR) rate was only 5%. In another report, only 9% of patients treated with a regimen combining cetuximab with capecitabine, oxaliplatin, and preoperative radiation therapy achieved a pCR.[11]



		T0	T1		T2
Tissue	*kRAS* mutations	X			
	cDNA microarrays	X	X		
	IHC: EGFR & Ki67	X	X		X
	Pathologic response				X
	Fibro-inflammation				X
Blood	Proteomics	X	X		
	ELISAs	X	X		X

Fig 1. Design of the clinical study and overview of the experiments performed on plasma and tumor biopsies. Tumor biopsies and plasma samples were taken at baseline (T0), 1 week after an initial dose of cetuxmab but before the start of chemoradiotherapy (T1), and at the time of surgery (T2). IHC, immunohistochemistry; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay.

These data contrast with the 16% pCR rate observed by this group when they used the same regimen without cetuximab.[12] Although nonrandomized, these two trials raise the question of how to optimally combine cetuximab with CRT and highlight the need for a better understanding of the molecular mechanisms involved. We investigated the molecular responses of patients in our phase II clinical study.[10] We show that cetuximab alone has an important impact on tumor cell proliferation and inflammation as well as the release of EGFR ligands. Our results also point to some biomarkers that might predict the efficacy of cetuximab-based CRT.

PATIENTS AND METHODS

Patients

Forty-one patients with rectal cancer (T3-4 and/or N+) were enrolled onto a phase I/II study with preoperative capecitabine in combination with cetuximab between November 2004 and June 2006 (Fig 1). Details of the eligibility criteria, pretreatment evaluation, radiotherapy, chemotherapy, cetuximab administration, and surgery have been published.[10] The translational and clinical parts of the study were approved by the Independent Ethics Committee and Belgian Health authority in accordance with European Regulations and conducted in accordance with the Declaration of Helsinki (October 2000). The translational research was prospectively planned, and patients gave informed consent for repeated biopsies.

Blood and Tissue Samples

Tumor biopsies and blood were taken at three time points: baseline samples (T0), after the initial dose of cetuximab but before the start of CRT (T1), and at the time of surgery (T2) (Fig 1). At each time point, one biopsy was frozen and another biopsy was fixed in 4% formalin for paraffin embedding. The surgical specimen was routinely processed for tumor staging. Tumor response was assessed as described before[10] by pCR and regression grading according to Dworak et al[13] (grade 0, no regression; grade 1, minimal regression; grade 2, moderate regression; grade 3, good regression; and grade 4, total regression) and Wheeler et al[14] (grade 1, sterilization or only microscopic foci of adenocarcinoma remaining with marked fibrosis; grade 2, marked fibrosis but macroscopic disease; and grade 3, little or no fibrosis with abundant macroscopic disease). Similarly to Shia et al,[15] stromal responses in the resection specimens were scored as fibrotic type (fibrosis/sclerosis with sparse inflammatory cell component, comprising $< 25\%$ of the entire stroma) and fibroinflammatory type (fibrosis/sclerosis with a prominent inflammatory component comprising $> 25\%$ of the entire stroma). The regression grading and the stromal responses were assessed independently by two pathologists (C.S. and S.A.). In case of discrepancies ($< 10\%$), a consensus was obtained.

Enzyme-Linked Immunosorbent Assays

Plasma was assayed for EGF ligands by sandwich enzyme-linked immunosorbent assay (ELISA) following the instructions of the EGF ELISA kit

(DEG00; R&D Systems, Minneapolis, MN) and the transforming growth factor-α (TGF-α) ELISA kit (DTGA00; R&D Systems).

Immunohistochemistry

Five micrometer serial sections were stained by hematoxylin and eosin to identify tumor and immunohistochemically for EGFR (Ventana Medical Systems, Inc, Tucson, AZ; 3C6 clone) and Ki67 (RM 9106-RZ; Neomarkers, Fremont, CA) according to the manufacturer's instructions.

Scoring of the Images

Sections stained for Ki67 or EGFR were analyzed at a total magnification of $\times$200 field-by-field, from top left to bottom right. The mean of percentage of tumor cells positive for EGFR (membranous) or Ki67 (nuclei) was calculated for the different fields. Upregulation was defined as an increase of more than 5% positivity. To insure that the EGFR and Ki67 stains were correctly scored, different slides were reviewed by an independent observer, and no significant difference was found (Wilcoxon-matched pair test, $P = .9$, $P = .5$).

Proteomics

Levels of 96 proteins (Appendix Table A1, online only) known to be involved in cancer were analyzed in a Luminex 100 instrument (Luminex Corp, Austin, TX) and interpreted using proprietary data analysis software developed at Rules-Based Medicine (Austin, TX) and licensed to Qiagen Instruments (Qiagen, Santa Clarita, CA). Proteins that did not have values greater than the detection limit in more than 20% of the samples were excluded from the analysis. These were calcitonin, epiregulin, erythropoietin, interleukin (IL) -1α, IL-2, and matrix metalloproteinase (MMP) -9 at T0, calcitonin, epiregulin, IL-1α, and IL-2 at T1, and calcitonin, epiregulin, glutathione S-transferase, IL-1α, IL-2, and MMP-9 at T2.

Microarrays

After checking the concentration (Nanodrop; Thermo Scientific, Wilmington, DE) and quality (Agilent Bioanalyzer 2100; Agilent, Santa Clara, CA) of extracted RNA, RNA samples of high quality (RNA integrity number > 5) were hybridized to Affymetrix GeneChip HG-U133 Plus 2.0 and subsequently scanned in the GeneChip Scanner (Affymetrix, Santa Clara, CA). Quality control was performed using the Affymetrix GCOS software and the Bioconductor software package affyPLM. The Bioconductor package RMA was used for preprocessing the microarray data (Bioconductor, Seattle, WA).[16]

Next, a total of 54,613 probe sets was reduced to 27,650 genes by mapping the probe sets on Entrez Gene IDs by taking the median of all probe sets for the same gene. Probe sets that matched on multiple genes were excluded, and unknown probe sets were given an arbitrary Entrez Gene ID. Finally, taking into account the low signal-to-noise ratio of microarray data, a prefiltering without reference to phenotype was used to retain the 6,913 genes (25%) with the highest variation across all samples.

DNA Extraction and k-ras Mutation Analysis

DNA was extracted from the pretreatment paraffin blocks by a phenol-chloroform-isoamylic alcohol (25:24:1) extraction, followed by a chloroform-isoamylic alcohol (24:1) and a sodium acetic acid (3 mol/L, pH 5.2) precipitation. An allele-specific Taqman polymerase chain reaction was used to screen for the seven most frequent mutations in codons 12 and 13 of the *k-ras* gene.[17]

Statistical Analysis

Differences in expression of proteins with time and their correlation with response was determined by a Wilcoxon rank sum or Kruskal-Wallis test, where appropriate. Kaplan-Meier analyses were used to calculate disease-free survival (DFS) probabilities and a log-rank test was used to compare groups. A multivariate logistic regression model was used to assess the independent effect of cetuximab on markers. All the tests were two-sided, with $P < .05$ for significance.

A sign-rank test (MATLAB; The Mathworks, Inc, Natick, MA) was used to determine the significance of microarray and proteomic data after cetuximab treatment. Levels of $P < .0005$ and $P < .05$ were considered as significant for the microarray and proteomics data, respectively, the difference being a result of dimensionality. The ability of proteomic data to predict outcome was analyzed using least squares support vector machines models, as described before.[18] For microarray data, a clustered prediction analysis for microarrays analysis[19] identified a minimal subset of genes that succinctly characterized patient groups with different responses to cetuximab. To determine the pathways most affected by cetuximab, a gene-enrichment analysis was performed with the DAVID-EASE program.[20]

RESULTS

Updated Pathologic and Clinical Results

Patient characteristics were described in an initial report.[10] Three patients were not assessable because they did not undergo surgery (disease progression, n = 1; death, n = 1; and unresectable disease found at surgery, n = 1). Pathologic TNM classification showed downstaging in 15 (39%) of 38 patients. Only two patients had a pCR (5%). The Dworak regression grades were distributed as follows: grade 0 (0%), grade 1 (11%), grade 2 (58%), grade 3 (26%), and grade 4 (5%). Wheeler grade 1, 2, and 3 regression was found in 71%, 26%, and 3% of patients, respectively. Forty-six percent of surgical specimens had a marked inflammatory cell component, whereas 54% had a predominantly fibrotic type stromal response by Shia's[15] critieria. A mutation in the *k-ras* gene was identified in 31% (12 of 39) of cases.

The median follow-up time was 32 months (range, 4.8 to 46.2 months). Local relapses and distant metastases were recorded in three (7%) and eight (20%) patients, respectively. Of the three patients with local relapses, only one patient developed distant metastases. Median DFS has not yet been reached, but at 2 years, DFS was 78%.

Impact of Cetuximab Monotherapy on the Tumor

Tumor biopsies obtained at baseline and after a single loading dose of cetuximab were compared using gene microarrays and immunohistochemistry. Microarray analysis identified 16 genes as significantly ($P < .0005$) influenced by cetuximab. Of these, three were involved in proliferation (*PIK3R1*, *CGREF1*, *PLAGL1*), and three others were involved in tumor invasion (*SERPINE2*, *TNS4*, *S100A6*; Table 1). Ki67 staining to measure changes in tumor proliferation showed a decrease in median expression from 85% to 67% ($P = .0002$; Fig 2A) after the loading dose of cetuximab, whereas EGFR expression was upregulated in 55% of cases, downregulated in 30% (10 of 33), and remained unchanged in 15% (five of 33).

Impact of Cetuximab Monotherapy on Plasma Proteins

Plasma samples obtained at baseline and after the loading dose of cetuximab were compared using xMAP technology (Luminex Corp). Levels of 13 proteins were significantly modified ($P < .05$; Table 1). The EGFR ligands, TGF-α and amphiregulin, were upregulated, although EGF expression was not modified and plasma EGFR levels decreased. To confirm the results of the Luminex analysis, ELISAs were performed for TGF-α and EGF. Cetuximab treatment increased TGF-α concentration in 73% (29 of 40) of patients ($P < .001$; Fig 2B), but EGF levels did not significantly change ($P = .12$). When multivariate logistic regression analyses of the gene and protein data obtained from tumor and plasma were performed, increases in EGFR ($P < .0001$) and plasma TGF-α ($P = .03$) after cetuximab treatment remained highly significant.

The other proteins upregulated by cetuximab were involved in inflammation (IL-1ra, IL-18, MDC, TNFR-II, MIP-1b, and ICAM-1) and lipid metabolism (adiponectin, ApoA-I, and Apo H).

Table 1. The 16 Genes and 13 Proteins Most Significantly Influenced by the Cetuximab Initial Dose

Gene or Protein	Full Name	Regulation	Function
Genes			
AIM1L	Absent in melanoma 1-like	Down	No known tumor-related function
C6orf141	Chromosome 6 open reading frame 141	Down	No known tumor-related function
SERPINE2	Serpin peptidase inhibitor, clade E	Down	Invasion
C18orf37	Chromosome 18 open reading frame 37	Down	No known tumor-related function
HKR1	GLI-Kruppel family member HKR1	Up	No known tumor-related function
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	Up	Proliferation, adherence, transformation, and survival
CGREF-1	Cell growth regulator with EF-hand domain 1	Down	Proliferation
PLAGL1	Pleiomorphic adenoma gene-like 1	Up	Proliferation, tumor suppressor gene
S100 A6	S100 calcium binding protein A6	Down	Cell cycle progression, invasion
FAM57A	Family with sequence similarity 57, member A	Down	No known tumor-related function
FLJ32252	Hypothetical protein FLJ32252	Down	No known tumor-related function
ZNF207	Zinc finger protein 207	Up	No known tumor-related function
IL33	Interleukin 33	Down	Inflammation
OCC-1	Overexpressed in colon carcinoma-1	Down	Cancer marker
EPM2AIP1	EPM2A (laforin) interacting protein 1	Up	No known tumor-related function
TENSIN 4	Tensin 4	Down	Cell adhesion molecule, invasion
Proteins			
EGFR	Epidermal growth factor receptor	Down	EGFR
TGF-α	Transforming growth factor-alpha	Up	EGFR ligand
ICAM-1	Intercellular adhesion molecule-1	Up	Inflammation
ARE	Amphiregulin	Up	EGFR ligand
IL-1ra	Interleukin 1 receptor antagonist	Up	Inflammation
IL-18	Interleukin 18	Up	Inflammation
Adiponectin	Adiponectin	Down	Lipid metabolism
ApoA-I	Apolipoprotein A1	Down	Lipid metabolism
MDC	Macrophage-derived chemokine	Up	Inflammation
Apo H	Apolipoprotein H	Down	Lipid metabolism
TNFR-II	Tumor necrosis factor receptor II	Up	Inflammation
MIP-1β	Macrophage inflammatory protein-1β	Up	Inflammation
PAP	Prostatic acid phosphatase	Down	Cancer marker

NOTE. For gene expression and proteomics, $P < .0005$ and $P < .05$ were used as cut-off values, respectively.

Correlation Between Biomarkers and Outcome

The above-mentioned biomarkers were analyzed for their association with pathologic response and DFS. Tumor downstaging was associated with upregulated TGF-α ($P = .05$) and downregulated Ki67 expression ($P = .01$) after the cetuximab loading dose. A similar, although not significant, association was seen with Dworak regression criteria (TGF-α, $P = .24$; Ki67, $P = .24$), whereas for Wheeler regression grade, only a trend with TGF-α expression ($P = .14$) was observed. Expression of EGF in the plasma, EGFR in the tumor, and *k-ras* mutation did not predict the pathologic response to CRT. Wild-type *k-ras* tumors tended to show regression using the Wheeler ($P = .09$) but not for the Dworak ($P = .36$) criteria and showed no correlation with tumor downstaging ($P = .69$). In summary, proteomic and microarray analyses did not identify simple predictive signatures for pathologic response, as has been reported elsewhere.[18] In contrast, DFS of patients was better if the initial dose of cetuximab upregulated EGFR in the tumor ($P = .02$) or if there were fibro-inflammatory changes in the resected specimen ($P = .03$; Fig 3).

Proteomic analysis showed that changes in expression of six proteins after the cetuximab initial dose (IgM, IL-4, tumor necrosis factor β [TNF-β], adiponectin, growth hormone, and thrombopoietin) could predict the occurrence of local recurrences and/or distant metastases with an accuracy of 83.3%, a sensitivity of 50%, and a specificity of 93%. In patients with recurrences, growth hormone, IgM, thrombopoietin, and TNF-β were upregulated, IL-4 was downregulated, and adiponectin showed less of a decrease. Furthermore, PAM analysis of microarray data identified a subset of genes before (50 genes) and after (40 genes) cetuximab administration that characterizes patient groups with different relapse potential (Appendix Tables A2 and A3, online only). Pretreatment high levels of expression of genes mainly involved in extracellular matrix functions (eg, collagen, asporin, fibulin, fibrillin, actin, and MMP11), or metabolism (eg, *IGFBP3, CPXM1, CPE,* and *AEBP1*) were found in patients who experienced relapse (Appendix Table A2). After one dose of cetuximab, most of the genes upregulated were related to the inflammatory response (immunoglobulin, MHC-I, IL-8, CD8, CD27, and so on) and in patients with no recurrences (Appendix Table A3). Using DAVID-EASE analysis,[20] we could conclude that 38% of the pathways upregulated in patients without recurrences were related to inflammation, and this increased to 45% of the pathways if the enriched terms with a *P* value below .05 were taken into account (Appendix Table A4, online only). These results, together with the proteomics results and the histologic analyses for fibro-inflammatory changes, confirm the importance of the inflammatory response in prediction of response to this treatment.

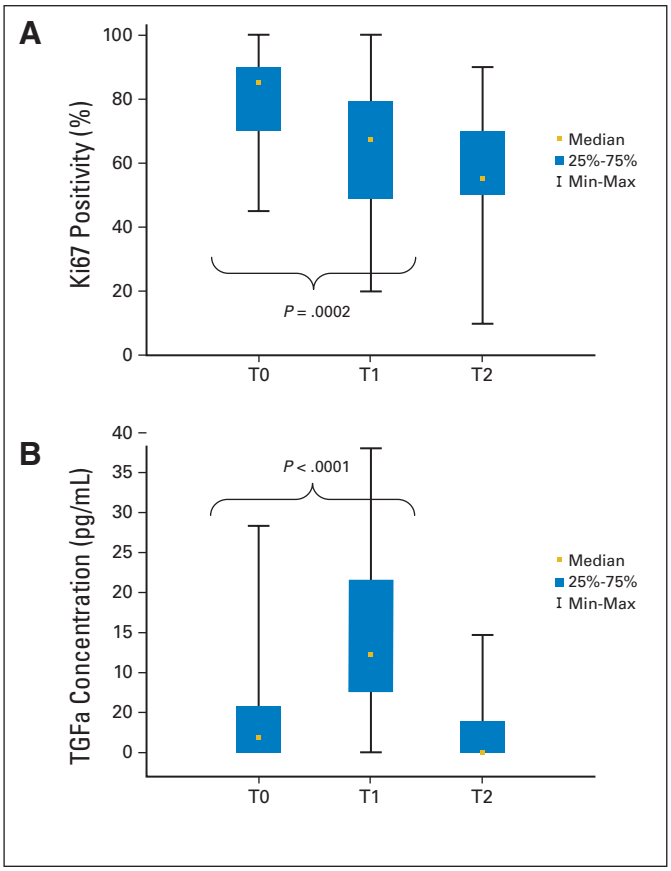


Fig 2. (A) Ki67 expression (assessed by immunohistochemistry) in tumor tissue and (B) transforming growth factor-α (TGF-α) expression (assessed by enzyme-linked immunosorbent assay) in plasma at three different time points: before treatment (T0), after cetuximab initial dose (T1), and at surgery (T2). Ki67 and TGF-α expression are significantly different at T1. Min, minimum; Max, maximum.

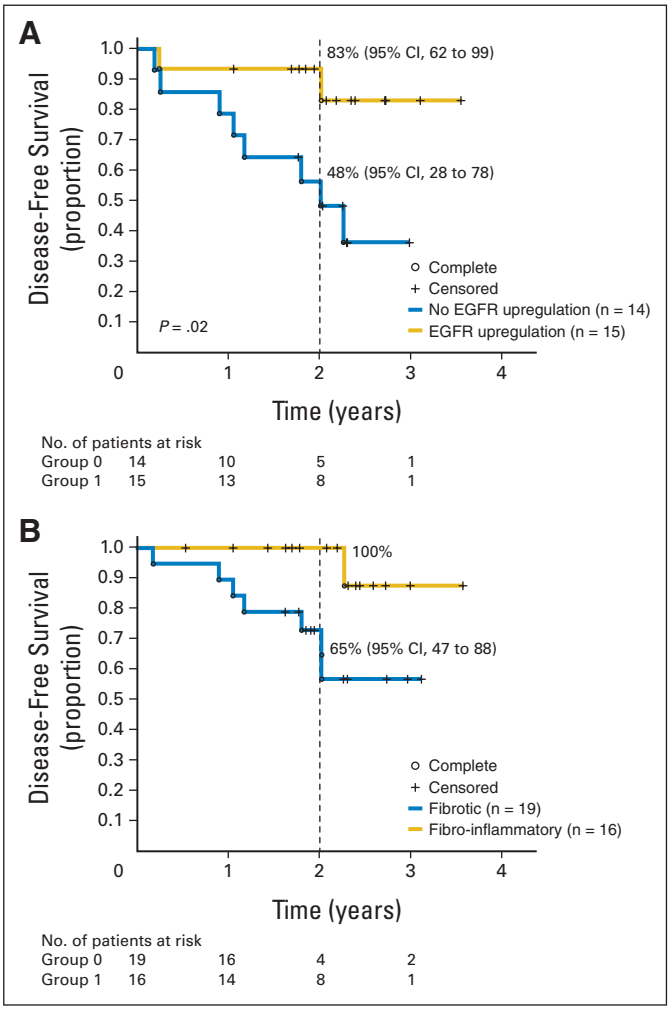


Fig 3. Disease-free survival (DFS) (A) for patients with and without upregulation of epidermal growth factor receptor (EGFR) in the tumor after the cetuximab initial dose (P = .02) and (B) fibro-inflammatory changes in the resected specimen (P = .03). The cumulative survival at 2 years (+95% CIs) is also indicated on the figure.

DISCUSSION

The design of this study gave us a unique opportunity to investigate the molecular effect of a single loading dose of cetuximab on untreated primary rectal tumors and to identify potential biomarkers that should be investigated further for their ability to predict efficacy of preoperative CRT or cetuximab-based therapy.

Proteomics revealed that cetuximab treatment alone increased expression of proinflammatory proteins, decreased those involved in lipid metabolism, and caused release of some EGFR ligands. This could be important because tumor infiltration by inflammatory cells seems to predict a better outcome after CRT,[15,21] and lipogenesis is clearly related to tumor development and growth.[22] Interestingly, plasma TGF-α, but not EGF or EGFR, levels were upregulated in almost all patients after the initial cetuximab dose. Increased levels of TGF-α might block EGFR and serve as a good predictor of response because it was correlated with T downstaging in our study. Similarly, mRNA expression of epiregulin and amphiregulin in tumor was found to be correlated with DFS in patients with metastatic colorectal cancer treated with cetuximab monotherapy.[24] In our study, epiregulin was excluded from the analysis because of low detection levels. Although we did see upregulation of amphiregulin, this did not correlate with response. These discrepancies may be because we measured plasma protein levels, whereas Khambata-Ford et al[24] examined tumor mRNA.

Our data agree with recent conclusions that EGFR expression assessed immunohistochemically is not correlated with response to treatment.[7,25-28] However, it is of interest that patients whose tumors upregulated EGFR after the first dose of cetuximab had significantly better DFS. This contrasts with the evidence that activation of EGFR pathways causes resistance to preoperative CRT regimens.[29-31] We hypothesize that this upregulation could be a salvage response of the tumor that could make more EGFR available as a target for cetuximab. Clearly, the dynamics of these ligand-receptor interactions are complex and need to be considered in future clinical trials.

One aim of this investigation was to determine the basis for the apparently relatively low pCR in patients receiving cetuximab along with CRT.[11,32] A likely explanation is that the pre-CRT initial dose of cetuximab significantly decreased tumor cell proliferation, as shown by Ki67 expression and the microarray data. Because capecitabine needs to be taken up by proliferating cells to exert its cytotoxic and

radiosensitizing properties, the chemotherapy in the CRT regimen might have been compromised by cetuximab pretreatment. This is supported by findings by at least two different groups showing that elevated tumor proliferation in rectal cancer cells before or after CRT is associated with a better response and improved DFS.[33,34] Cetuximab might be more effective if it is not started before CRT, if it is combined with radiotherapy in the absence of chemotherapy, or if it is given after CRT as maintenance therapy.[35]

In addition to the therapeutic sequence, selection of patients for cetuximab treatment seems to be important for outcome in colorectal cancer. Recent data in metastasized colorectal cancer suggest that *k-ras* mutations confer resistance to this agent.[36,37] In our study, tumors with *k-ras* mutations had no significantly worse response to CRT combined with cetuximab, but the number of patients was small, and we cannot exclude the possibility that selection of a population enriched for wild-type *k-ras* tumors might show more effect. However, preliminary results from another study in rectal cancer indicate only a trend for better response in patients without *k-ras* mutations to CRT plus cetuximab, which is in agreement with our data.[32]

Finally, our proteomics and microarray analyses suggested that genes involved in extracellular matrix functions, metabolism, and inflammatory response were important for systemic or local relapse. These data should be interpreted as exploratory and with caution because they were generated on a limited number of patients treated with a nonstandard preoperative CRT regimen. However, the finding that the inflammatory response to treatment seemed to be important was consistent across the different molecular investigations that we performed and was further confirmed by examination of the surgical specimens where a predominant fibro-inflammatory status was associated with better DFS, as suggested by Shia et al.[15] This reinforces the concept that host response to therapy could be an important prognostic factor in rectal cancer.

In conclusion, our work identified potential molecular pathways involved in cetuximab response in patients with colorectal cancer that should be investigated further to determine their ability to predict clinical outcome in a laboratory-driven larger randomized trial. However, our data suggest that future trials should be designed to combine cetuximab with radiotherapy alone or administer cetuximab after or during CRT rather than before CRT to avoid its antiproliferative effects interfering with the outcome.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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REFERENCES

1. Bosset JF, Calais G, Mineur L, et al: Enhanced tumorocidal effect of chemotherapy with preoperative radiotherapy for rectal cancer: Preliminary results—EORTC 22921. J Clin Oncol 23: 5620-5627, 2005
2. Cammà C, Giunta M, Fiorica F, et al: Preoperative radiotherapy for resectable rectal cancer: A meta-analysis. JAMA 284:1008-1015, 2000
3. Dahlberg M, Glimelius B, Pahlman L: Improved survival and reduction in local failure rates after preoperative radiotherapy: Evidence for the generalizability of the results of Swedish Rectal Cancer Trial. Ann Surg 229:493-497, 1999
4. Gérard JP, Conroy T, Bonnetain F, et al: Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: Results of FFCD 9203. J Clin Oncol 24:4620-4625, 2006
5. Martling A, Holm T, Johansson H, et al: The Stockholm II trial on preoperative radiotherapy in rectal carcinoma: Long-term follow-up of a population-based study. Cancer 92:896-902, 2001
6. Chung KY, Saltz LB: Antibody-based therapies for colorectal cancer. Oncologist 10:701-709, 2005
7. Cunningham D, Humblet Y, Siena S, et al: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 351:337-345, 2004
8. Jonker DJ, O'Callaghan CJ, Karapetis CS, et al: Cetuximab for the treatment of colorectal cancer. N Engl J Med 357:2040-2048, 2007
9. Bonner JA, Harari PM, Giralt J, et al: Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 354:567-578, 2006
10. Machiels JP, Sempoux C, Scalliet P, et al: Phase I/II study of preoperative cetuximab, capecitabine, and external beam radiotherapy in patients with rectal cancer. Ann Oncol 18:738-744, 2007
11. Rödel C, Arnold D, Hipp M, et al: Phase I-II trial of cetuximab, capecitabine, oxaliplatin, and radiotherapy as preoperative treatment in rectal cancer. Int J Radiat Oncol Biol Phys 70:1081-1086, 2008
12. Rödel C, Liersch T, Hermann RM, et al: Multicenter phase II trial of chemoradiation with oxaliplatin for rectal cancer. J Clin Oncol 25:110-117, 2007
13. Dworak O, Keilholz L, Hoffmann A: Pathological features of rectal cancer after preoperative radiochemotherapy. Int J Colorectal Dis 12:19-23, 1997
14. Wheeler JM, Warren BF, Mortensen NJ, et al: Quantification of histologic regression of rectal cancer after irradiation: A proposal for a modified staging system. Dis Colon Rectum 45:1051-1056, 2002
15. Shia J, Guillem JG, Moore HG, et al: Patterns of morphologic alteration in residual rectal carcinoma following preoperative chemoradiation and their association with long-term outcome. Am J Surg Pathol 28:215-223, 2004
16. Gentleman RC, Carey VJ, Bates DM, et al: Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol 5:R80, 2004
17. De Roock W, Piessevaux H, De Schutter J, et al: KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann Oncol 19:508-515, 2008
18. Daemen A, Gevaert O, De Bie T, et al: Integrating microarray and proteomics data to predict the response on cetuximab in patients with rectal cancer. Pac Symp Biocomput 166-177, 2008

19. Tibshirani R, Hastie T, Narasimhan B, et al: Diagnosis of multiple cancer types by shrunken centroids of gene expression. Proc Natl Acad Sci U S A 99:6567-6572, 2002

20. National Institute of Allergy and Infectious Diseases, National Institutes of Health: The Database for Annotation, Visualization and Integrated Discovery. http://david.abcc.ncifcrf.gov/

21. Debucquoy A, Libbrecht L, Roobrouck V, et al: Morphological features and molecular markers in rectal cancer from 95 patients included in the European Organisation for Research and Treatment of Cancer 22921 trial: Prognostic value and effects of preoperative radio (chemo) therapy. Eur J Cancer 44:791-797, 2008

22. Swinnen JV, Brusselmans K, Verhoeven G: Increased lipogenesis in cancer cells: New players, novel targets. Curr Opin Clin Nutr Metab Care 9:358-365, 2006

23. Reference deleted

24. Khambata-Ford S, Garrett CR, Meropol NJ, et al: 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

25. Baselga J, Trigo JM, Bourhis J, et al: Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck. J Clin Oncol 23:5568-5577, 2005

26. Saltz LB, Meropol NJ, Loehrer PJ Sr, et al: Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 22:1201-1208, 2004

27. Chung KY, Shia J, Kemeny NE, et al: Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23:1803-1810, 2005

28. Hebbar M, Wacrenier A, Desauw C, et al: Lack of usefulness of epidermal growth factor receptor expression determination for cetuximab therapy in patients with colorectal cancer. Anticancer Drugs 17:855-857, 2006

29. Azria D, Bibeau F, Barbier N, et al: Prognostic impact of epidermal growth factor receptor (EGFR) expression on loco-regional recurrence after preoperative radiotherapy in rectal cancer. BMC Cancer 5:62, 2005

30. Bertolini F, Bengala C, Losi L, et al: Prognostic and predictive value of baseline and posttreatment molecular marker expression in locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy. Int J Radiat Oncol Biol Phys 68:1455-1461, 2007

31. Giralt J, de las HM, Cerezo L, et al: The expression of epidermal growth factor receptor results in a worse prognosis for patients with rectal cancer treated with preoperative radiotherapy: A multicenter, retrospective analysis. Radiother Oncol 74:101-108, 2005

32. Bengala C, Betteli S, Bertolini F, et al: Predictive value of EGFR gene copy number and K-ras mutation for pathological response to preoperative cetuximab, 5-FU, and radiation therapy in locally advanced rectal cancer (LARC). J Clin Oncol 26: 209s, 2008 (suppl; abstr 4125)

33. Rau B, Sturm I, Lage H, et al: Dynamic expression profile of p21WAF1/CIP1 and Ki-67 predicts survival in rectal carcinoma treated with preoperative radiochemotherapy. J Clin Oncol 21: 3391-3401, 2003

34. Rödel C, Grabenbauer GG, Papadopoulos T, et al: Apoptosis as a cellular predictor for histopathologic response to neoadjuvant radiochemotherapy in patients with rectal cancer. Int J Radiat Oncol Biol Phys 52:294-303, 2002

35. Milas L, Fang FM, Mason KA, et al: Importance of maintenance therapy in C225-induced enhancement of tumor control by fractionated radiation. Int J Radiat Oncol Biol Phys 67:568-572, 2007

36. Di Fiore F, Blanchard F, Charbonnier F, et al: Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 96:1166-1169, 2007

37. Lièvre A, Bachet JB, Boige V, et al: KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 26:374-379, 2008

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