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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

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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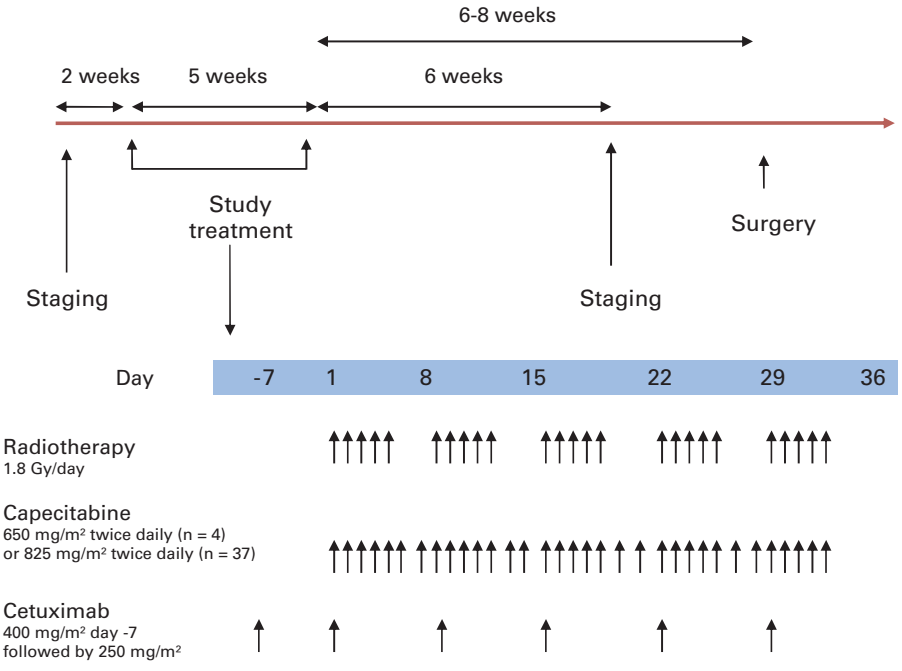
INTRODUCTION

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]

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		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

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(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).

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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.

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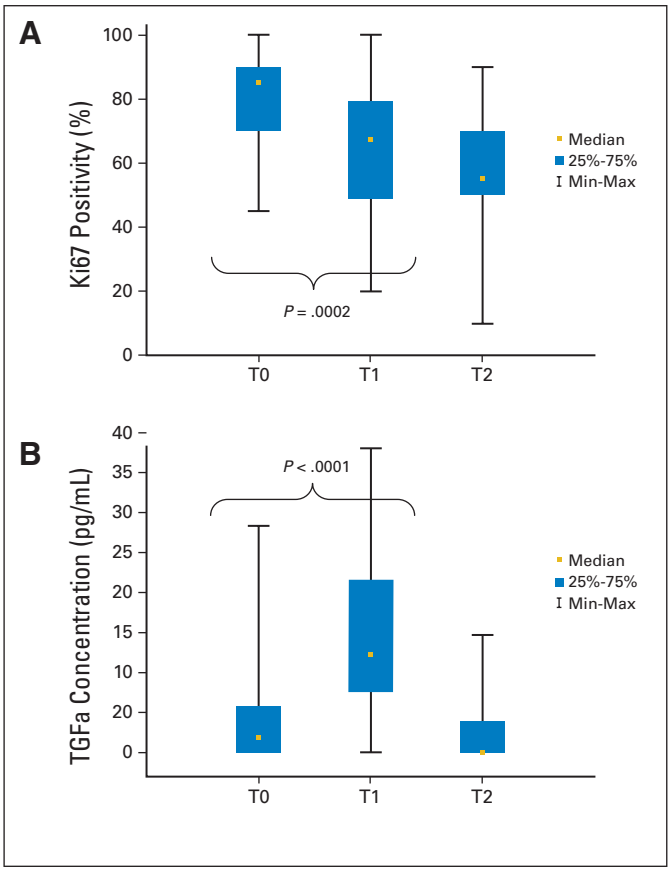


Fig 2. (A) Ki67 expression (assessed by immunohistochemistry) in tumor tissue and (B) transforming growth factor-α (TGF-α) expression (assessed by enzyme-linked immunosorbent assay) in plasma at three different time points: before treatment (T0), after cetuximab initial dose (T1), and at surgery (T2).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

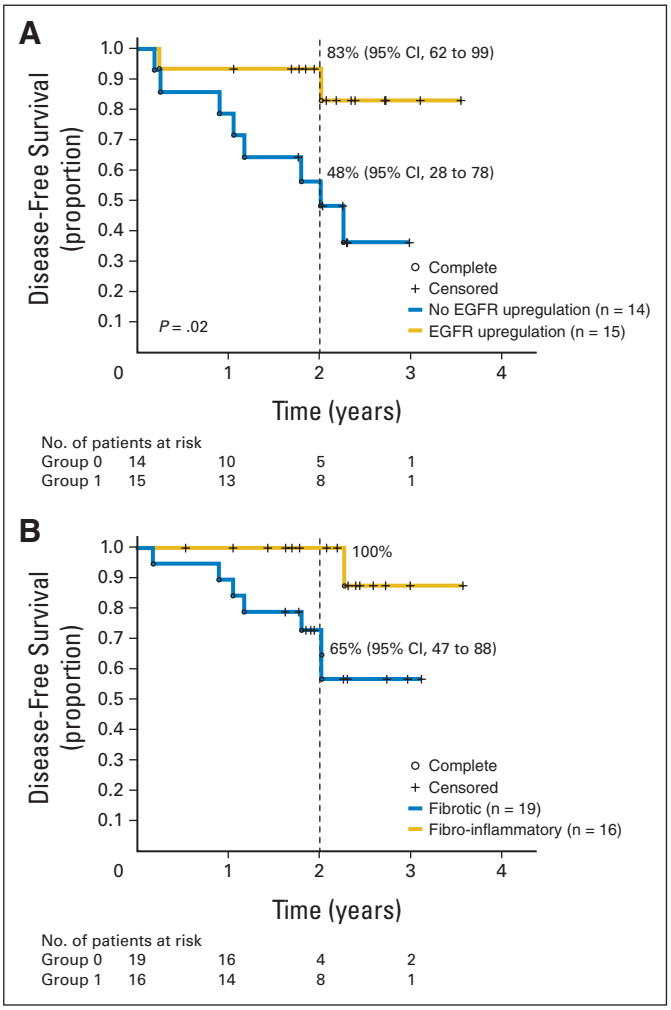


Fig 3. Disease-free survival (DFS) (A) for patients with and without upregulation of epidermal growth factor receptor (EGFR) in the tumor after the cetuximab initial dose (P = .02) and (B) fibro-inflammatory changes in the resected specimen (P = .03). The cumulative survival at 2 years (+95% CIs) is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

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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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Appendix

Table A1. List of the 96 Proteins Used in the Proteomics Analysis

No.	Protein	No.	Protein
1	α-1 Antitrypsin	49	IL-1α
2	Adiponectin	50	IL-1β
3	α-2 Macroglobulin	51	IL-1ra
4	α-Fetoprotein	52	IL-2
5	Amphiregulin	53	IL-3
6	Apolipoprotein A1	54	IL-4
7	Apolipoprotein CIII	55	IL-5
8	Apolipoprotein H	56	IL-6
9	β-2 Microglobulin	57	IL-7
10	Brain-derived neurotrophic factor	58	IL-8
11	Complement 3	59	Insulin
12	Cancer antigen 125	60	Leptin
13	Cancer antigen 19-9	61	Lipoprotein (a)
14	Calcitonin	62	Lymphotactin
15	CD40	63	MCP-1
16	CD40 ligand	64	MDC
17	Carcinoembryonic antigen	65	MIP-1α
18	Creatine kinase-MB	66	MIP-1β
19	C Reactive protein	67	MMP-2
20	EGF	68	MMP-3
21	ENA-78	69	MMP-9
22	Endothelin-1	70	Myeloperoxidase
23	EN-RAGE	71	Myoglobin
24	Eotaxin	72	PAI-1
25	Epiregulin	73	Prostatic acid phosphatase
26	Erythropoietin	74	PAPP-A
27	Fatty acid binding protein	75	Prostate-specific antigen, free
28	Factor VII	76	RANTES
29	Ferritin	77	Serum amyloid P
30	FGF basic	78	Stem cell factor
31	Fibrinogen	79	SGOT
32	G-CSF	80	SHBG
33	Growth hormone	81	Thyroxine-binding globulin
34	GM-CSF	82	Tissue factor
35	Glutathione S-transferase	83	TGF-α
36	Haptoglobin	84	TIMP-1
37	ICAM-1	85	TNF RII
38	IgA	86	TNF-α
39	IgE	87	TNF-β
40	IGF-1	88	Thrombopoietin
41	IgM	89	Thyroid-stimulating hormone
42	IL-10	90	Thrombospondin-1
43	IL-12p40	91	VCAM-1
44	IL-12p70	92	VEGF
45	IL-13	93	von Willebrand Factor
46	IL-15	94	Betacellulin
47	IL-16	95	EGFR
48	IL-18	96	HB-EGF

Abbreviations: IL, interleukin; MCP-1, monocyte chemotactic protein-1; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; EGF, epidermal growth factor; ENA-78, epithelial neutrophil activating protein-78; EN-RAGE, receptor for advanced glycation end products; PAI-1, plasminogen activator inhibitor-1; PAPP-A, pregnancy associated plasma protein-A; RANTES, chemokine (C-C motif) ligand 5; SGOT, serum glutamic oxaloacetic transaminase; G-CSF, granulocyte colony-stimulating factor; SHBG, sex hormone-binding globulin; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF, transforming growth factor; ICAM-1, intercellular adhesion molecule-1; TNF, tumor necrosis factor; Ig, immunoglobulin; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGF-like growth factor.

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Table A2. Fifty Most Significant Genes at T0

No.	Entrez Gene ID	Gene Name	0-Score	1-Score
1	1311	Cartilage oligomeric matrix protein	−0.0996	0.3485
2	6364	Chemokine (C-C motif) ligand 20	0.0881	−0.3085
3	6423	Secreted frizzled-related protein 2	−0.0752	0.2631
4	6424	Secreted frizzled-related protein 4	−0.0613	0.2147
5	27123	Dickkopf homolog 2	−0.0548	0.1917
6	81578	Collagen, type XXI, α 1.	−0.0416	0.1455
7	56265	Carboxypeptidase X (M14 family), member 1	−0.0412	0.1443
8	221091	LOC221091	−0.0397	0.1389
9	165	Adipocyte enhancer binding protein 1	−0.037	0.1295
10	54829	Asporin	−0.0333	0.1164
11	83468	Glycosyltransferase 8 domain containing 2	−0.0308	0.1079
12	2192	Fibulin 1	−0.03	0.105
13	3486	Insulin-like growth factor binding protein 3	−0.0298	0.1043
14	83690	Cysteine-rich secretory protein LCCL domain containing 1	−0.0285	0.0999
15	116039	Odd-skipped related 2	−0.0265	0.0926
16	1363	Carboxypeptidase E	−0.0262	0.0917
17	1307	Collagen, type XVI, alpha 1	−0.0254	0.0889
18	387914	Shisa homolog 2	−0.0238	0.0833
19	1833	Epiphycan	−0.0227	0.0795
20	25817	Family with sequence similarity 19 member A5	−0.0221	0.0775
21	8082	Sarcospan (Kras oncogene-associated gene)	−0.0208	0.0729
22	7292	Tumor necrosis factor (ligand) superfamily, member 4	−0.0203	0.0711
23	2200	Fibrillin 1	−0.02	0.0699
24	59	Actin, alpha 2, smooth muscle, aorta	−0.0195	0.0681
25	56034	Platelet derived growth factor C	−0.0193	0.0675
26	4969	Osteoglycin	−0.0177	0.0618
27	114928	G protein-coupled receptor associated sorting protein 2	−0.0151	0.0529
28	6695	Sparc/osteonectin	−0.0142	0.0497
29	151887	CCDC80	−0.0129	0.045
30	81031	Solute carrier family 2	−0.0114	0.0401
31	4256	Matrix Gla protein	−0.0105	0.0367
32	25925	Zinc finger protein 521	−0.0079	0.0278
33	2326	Flavin containing monooxygenase 1	−0.0076	0.0266
34	3671	IG superfamily containing leucine-rich repeat	−0.0074	0.0259
35	3400	Inhibitor of DNA binding 4	−0.0074	0.0259
36	91624	Nexilin (F actin binding protein)	−0.0071	0.0249
37	4320	Matrix metallopeptidase 11	−0.0071	0.0249
38	83,872	Hemicentin 1	−0.0068	0.0238
39	79901	Cytochrome b reductase 1	−0.0066	0.0232
40	23414	Zinc finger protein, multitype 2	−0.0066	0.0231
41	114902	C1q and tumor necrosis factor related protein 5	−0.0065	0.0227
42	8532	Carboxypeptidase Z	−0.0057	0.0201
43	1296	Collagen, type VIII, alpha 2	−0.0034	0.0117
44	7980	Tissue factor pathway inhibitor 2	0.0028	−0.01
45	284262	Hypothetical protein LOC284262	−0.0026	0.0092
46	330	Baculoviral IAP repeat-containing 3	0.0025	−0.0088
47	8076	Microfibrillar associated protein 5	−0.002	0.0071
48	8777	Multiple PDZ domain protein	−0.0016	0.0058
49	8483	Cartilage intermediate layer protein	−7.00E-04	0.0025
50	1277	Collagen, type I, alpha 1	−7.00E-04	0.0023

NOTE. Table with the 50 most significant genes at baseline (T0), identified by the PAM analysis. The 0 score and 1 score show the median (normalized) increase or decrease in the group without and with recurrences, respectively.

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Table A3. Forty Most Significant Genes at T1

No.	Entrez Gene ID	Gene Name	0 Score	1 Score
1	3512	**Immunoglobulin J polypeptide**	0.0675	−0.2362
2	3934	Lipocalin 2	0.0557	−0.195
3	11221	Dual specificity phosphatase 10	−0.055	0.1924
4	1776	Deoxyribonuclease I-like 3	0.0546	−0.1911
5	608	**Tumor necrosis factor receptor superfamily, member 17**	0.052	−0.1819
6	284422	**Immunoglobulin heavy variable 3-23**	0.0459	−0.1607
7	939	**CD27 molecule**	0.0437	−0.1529
8	1830	Desmoglein 3	−0.0436	0.1527
9	28234	Solute carrier organic anion transporter family	−0.0432	0.1512
10	28902	**Immunoglobulin kappa variable 1D-13**	0.0423	−0.148
11	9934	Purinergic receptor P2Y	0.0414	−0.1448
12	3507	**Immunoglobulin heavy constant mu**	0.0393	−0.1376
13	5920	Retinoic acid receptor responder	0.0344	−0.1204
14	28,831	**Immunoglobulin lambda joining 3**	0.0335	−0.1172
15	6690	Serine peptidase inhibitor	0.033	−0.1155
16	3107	**Major histocompatibility complex, class I, C**	0.0306	−0.1071
17	29909	G protein-coupled receptor 171	0.03	−0.1052
18	834	Caspase 1	0.0284	−0.0996
19	1179	Chloride channel, calcium activated	0.0283	−0.0991
20	91353	**Immunoglobulin lambda-like polypeptide 3**	0.0261	−0.0915
21	124975	γ-glutamyltransferase 6	0.0237	−0.0829
22	11254	Solute carrier family 6	0.0223	−0.0779
23	646627	Phospholipase inhibitor	0.0216	−0.0756
24	10125	RAS guanyl releasing protein 1	0.0201	−0.0705
25	130367	Sphingosine-1-phosphate phosphotase 2	0.0185	−0.0646
26	83998	Regenerating islet-derived family	0.0181	−0.0634
27	914	**CD2 molecule**	0.0181	−0.0634
28	9920	Kelch repeat and BTB (POZ) domain containing 11	0.0173	−0.0606
29	23120	ATPase, class V, type 10B	0.0172	−0.0603
30	1359	Carboxypeptidase A3 (mast cell)	0.0165	−0.0578
31	3576	**Interleukin 8**	0.0154	−0.0538
32	3491	Cysteine-rich, angiogenic inducer, 61	−0.0153	0.0535
33	29966	Striatin, calmodulin binding protein 3	0.0152	−0.0531
34	10105	Peptidylprolyl isomerase F (cyclophilin F)	−0.0151	0.0527
35	6382	Syndecan 1	0.0147	−0.0514
36	51237	Hypothetical protein MGC29506	0.0146	−0.051
37	28755	**T cell receptor alpha constant**	0.0118	−0.0413
38	11072	Dual specificity phosphatase 14	−0.0105	0.0367
39	2053	Epoxide hydrolase 2, cytoplasmic	0.0101	−0.0353
40	6768	Suppression of tumorigenicity 14 (colon carcinoma)	0.0092	−0.0322

NOTE. Table with the 40 most significant genes after the initial dose of cetuximab but before the start of chemoradiotherapy (T1) identified by PAM analysis. The 0 score and 1 score show the median (normalized) increase or decrease in the group without and with recurrences, respectively. Genes in bold are involved in inflammation.

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Table A4. Functional Annotation Analysis With DAVID-EASE

Enriched Terms	% of Genes	*P*
GO:0002376~immune system process	26.47	.00009
GO:0006955~immune response	23.53	.00002
Disulfide bond	35.29	.00030
Direct protein sequencing	32.35	.00140
h_il17Pathway:IL 17 Signaling Pathway	8.82	.00146
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	8.82	.00218
GO:0048002~antigen processing and presentation of peptide antigen	8.82	.00262
GO:0045321~leukocyte activation	11.76	.00279
GO:0005886~plasma membrane	35.29	.00358
GO:0001775~cell activation	11.76	.00397
GO:0042612~MHC class I protein complex	8.82	.00420
GO:0051249~regulation of lymphocyte activation	8.82	.00567
GO:0044459~plasma membrane part	26.47	.00600
GO:0050865~regulation of cell activation	8.82	.00635
GO:0051239~regulation of multicellular organismal process	11.76	.00702
GO:0005887~integral to plasma membrane	20.59	.00733
GO:0031226~intrinsic to plasma membrane	20.59	.00780
Glycoprotein	35.29	.01020
GO:0000267~cell fraction	17.65	.01147
Hydrolase	20.59	.01281
Signal	29.41	.01517
Glycosylation site:N-linked (GlcNAc. . .)	32.35	.01628
GO:0003823~antigen binding	8.82	.02251
GO:0045619~regulation of lymphocyte differentiation	5.88	.02567
GO:0046649~lymphocyte activation	8.82	.02607
IPR003597:Immunoglobulin C1-set	8.82	.02828
GO:0050871~positive regulation of B cell activation	5.88	.02833
IPR003006:Immunoglobulin/major histocompatibility complex motif	8.82	.03197
GO:0043028~caspase regulator activity	5.88	.03319
SM00407:IGc1	8.82	.03514
GO:0050864~regulation of B cell activation	5.88	.03762
GO:0002274~myeloid leukocyte activation	5.88	.04025
GO:0005576~extracellular region	17.65	.04102
GO:0050896~response to stimulus	26.47	.04250
Topological domain:Extracellular	23.53	.04450
GO:0007242~intracellular signaling cascade	17.65	.04727
GO:0042611~MHC protein complex	8.82	.04756
GO:0007165~signal transduction	29.41	.04949
hsa04514:Cell adhesion molecules (CAMs)	8.82	.04951
GO:0051179~localization	26.47	.04993
h_thelperPathway:T Helper Cell Surface Molecules	5.88	.05044
GO:0019882~antigen processing and presentation	8.82	.05088
GO:0016502~nucleotide receptor activity	5.88	.05231
GO:0001608~nucleotide receptor activity, G-protein coupled	5.88	.05231
h_tcytotoxicPathway:T Cytotoxic Cell Surface Molecules	5.88	.05292
T cell	5.88	.05399
GO:0051050~positive regulation of transport	5.88	.05462
Surface antigen	5.88	.05534
IPR001368:TNFR/CD27/30/40/95 cysteine-rich region	5.88	.05741
GO:0044425~membrane part	41.18	.05776
GO:0048518~positive regulation of biological process	14.71	.05989
Transmembrane protein	11.76	.06060
SM00208:TNFR	5.88	.06496
Apoptosis	8.82	.06798
Pyroglutamic acid	5.88	.06870
GO:0016064~immunoglobulin mediated immune response	5.88	.07133
GO:0019724~B cell mediated immunity	5.88	.07388
GO:0051251~positive regulation of lymphocyte activation	5.88	.07515

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Table A4. Functional Annotation Analysis With DAVID-EASE (continued)

Enriched Terms	% of Genes	*P*
Transmembrane	32.35	.07625
GO:0007154~cell communication	29.41	.08316
GO:0004871~signal transducer activity	23.53	.08484
GO:0060089~molecular transducer activity	23.53	.08484
GO:0006915~apoptosis	11.76	.08896
GO:0042113~B cell activation	5.88	.09027
GO:0012501~programmed cell death	11.76	.09081
GO:0008283~cell proliferation	11.76	.09215
GO:0005624~membrane fraction	11.76	.09222
Signal peptide	23.53	.09230
Receptor	17.65	.09262
GO:0004872~receptor activity	20.59	.09561
GO:0002449~lymphocyte mediated immunity	5.88	.09650
Membrane	35.29	.09679
GO:0007243~protein kinase cascade	8.82	.09969

NOTE. Enriched terms upregulated after cetuximab loading dose in patients without relapses are shown. The enriched terms in bold are involved in inflammation (DAVID-EASE analysis).

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