Clinical Cancer Research

Gene Expression Markers of Efficacy and Resistance to Cetuximab Treatment in Metastatic Colorectal Cancer: Results from CALGB 80203 (Alliance)

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Abstract

Purpose: Formalin-fixed, paraffin-embedded tumor samples from CALGB 80203 were analyzed for expression of EGFR axis–related genes to identify prognostic or predictive biomarkers for cetuximab treatment.

Patients and Methods: Patients (238 total) with first-line metastatic colorectal cancer (mCRC) were randomized to FOLFOX or FOLFIRI chemotherapy ± cetuximab. qRT-PCR analyses were conducted on tissues from 103 patients at baseline to measure gene expression levels of HER-related genes, including amphiregulin (*AREG*), betacellulin (*BTC*), *NT5E* (*CD73*), *DUSP4*, *EGF*, *EGFR*, epigen (*EPGN*), epiregulin (*EREG*), *HBEGF*, *ERBB2* (*HER2*), *ERBB3* (*HER3*), *ERBB4* (*HER4*), *PHLDA1*, and *TGFA*. The interactions between expression levels and treatment with respect to progression-free survival (PFS) and overall survival (OS) were modeled using multiplicative Cox proportional hazards models.

Results: High tumor mRNA levels of *HER2* [hazard ratio (HR), 0.64; $P = 0.002$] and *EREG* (HR, 0.89; $P = 0.016$) were prognostic markers associated with longer PFS across all patients. *HER3* and *CD73* expression levels were identified as potential predictive markers of benefit from cetuximab. In *KRAS* wild-type (WT) tumors, low *HER3* expression was associated with longer OS from cetuximab treatment, whereas high *HER3* expression was associated with shorter OS from cetuximab treatment (chemo + cetuximab: HR, 1.15; chemo-only: HR, 0.48; $P_{interaction} = 0.029$). High *CD73* expression was associated with longer PFS from cetuximab treatment in patients with *KRAS*-WT (chemo + cetuximab: HR, 0.91; chemo-only: HR, 1.57; $P_{interaction} = 0.026$) and *KRAS*-mutant (Mut) tumors (chemo + cetuximab: HR, 0.80; chemo-only: HR, 1.29; $P = 0.025$).

Conclusions: Gene expression of *HER3* and *CD73* was identified as a potential predictive marker for cetuximab. These data implicate HER axis signaling and immune modulation as potential mechanisms of cetuximab action and sensitivity. *Clin Cancer Res; 21(5); 1078–86. ©2014 AACR.*

Introduction

Epidermal growth factor receptor (EGFR)–targeted therapies have shown clinical benefit in the treatment of numerous cancers, including metastatic colorectal cancer (mCRC; ref. 1). Cetuximab, a chimeric monoclonal anti-EGFR antibody, is FDA and EMA approved for use in combination with FOLFIRI chemotherapy in the first-line setting and as monotherapy or with irinotecan in late-line treatment of *KRAS* wild-type (WT) mCRC. Recent data also suggest the activity of cetuximab with FOLFOX-based chemotherapy (2).

EGFR is a member of the ERBB/HER family of receptor tyrosine kinases (RTK). Ligand binding causes homo- and hetero-dimerization between EGFR and the other members of the HER family (ERBB2/HER2, ERBB4/HER4, and the kinase-inactive ERBB3/HER3) resulting in downstream activation of the RAS–RAF–MEK and PI3K–AKT pathways (3). Multiple strategies have been developed for the therapeutic inhibition of EGFR signaling pathways and significant effort has been devoted to identifying biomarkers that can predict those patients most and least likely to benefit from EGFR-targeted therapies. Currently, only *RAS* mutation status has been validated as a predictive marker for anti-EGFR antibodies (4, 5). Activating *RAS* mutations occur downstream from the RTK EGFR, providing proliferative signals independent of EGFR ligand binding, and cause resistance to EGFR blockade (6, 7). The initial reports showing that mutations in *KRAS* conferred resistance to EGFR-targeting therapies focused on mutations in codons 12 and 13 of exon 2 (4, 8). Recent studies have identified mutations in exon 3 and 4 of KRAS and exons 2, 3, and 4 of NRAS as additional markers of resistance to anti-EGFR antibodies in colorectal cancer (9, 10). Intriguingly, gene expression signatures of activated *RAS* often indicate upregulation of several EGFR ligands and inflammatory mediators (11–13). Moreover, feedback loops

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-14-2313

Translational Relevance

Beyond *KRAS* status, there are no validated biomarkers for anti-EGFR therapy in metastatic colorectal cancer (mCRC). Expression of genes within the EGFR signaling axis has been reported to correlate with benefit, but most reports have used nonrandomized data that cannot distinguish prognostic and predictive markers. This report is one of the first generated from a randomized trial to identify predictive markers of benefit from cetuximab in mCRC. Gene expression of *HER3* and *CD73* was identified as a potential predictive marker for cetuximab. Although the current sample size is small and the conclusions should be considered preliminary, they implicate both HER axis signaling and immune modulation as potential mechanisms of cetuximab action and sensitivity.

involving EGFR have also been noted in the setting of RAF and MEK inhibition (14–16).

Other mutations of genes within the EGFR signaling pathway (*BRAF*, *PI3K*, loss of *PTEN* expression) do not consistently predict for benefit or resistance to anti-EGFR antibodies (17). Although less studied than common driver mutations, expression levels of nonmutated ligands and receptors have been reported as candidate predictors of benefit from cetuximab. High expression levels of two EGFR ligands, amphiregulin (*AREG*) and epiregulin (*EREG*), have been associated with longer progression-free survival (PFS) and higher response rates in *KRAS*-WT mCRC patients treated with cetuximab (13, 18, 19). Other markers associated with treatment outcome have also been identified, including ecto 5′-nucleotidase, *NT5E* (*CD73*; ref. 19). However, these biomarker analyses in cetuximab-treated mCRC patients were performed in nonrandomized clinical studies, necessitating further investigation and validation in randomized controlled trials.

The Cancer and Leukemia Group B (CALGB, now The Alliance for Clinical Trials in Oncology) 80203 trial was originally initiated as a phase III clinical trial of FOLFOX or FOLFIRI with or without cetuximab as first-line treatment of mCRC. However, with the FDA approval of bevacizumab for mCRC in 2004, CALGB 80203 was closed and its analysis plan was formally redesigned as a 1:1 randomized phase II study. Concurrently, the cooperative group then initiated CALGB 80405 to evaluate bevacizumab, cetuximab, and the combination of bevacizumab and cetuximab in a randomized phase III study. The clinical results for CALGB 80203 (20) and CALGB 80405 have been reported previously (2). There was no significant difference between the cetuximab and bevacizumab arms with respect to overall survival [OS;hazard ratio (HR), 0.925; 95% confidence interval (CI), 0.78–1.09; median OS 29.9 and 29.0 months, respectively] or PFS(HR, 1.04; 95% CI, 0.91–1.17; median PFS 10.4 and 10.8 months, respectively). These results again emphasize the need for further refinement of the individual patient populations and the development of new predictive biomarkers beyond *KRAS* status to improve patient outcomes.

To this end, we hypothesized that the gene expression of EGF signaling–related genes in colorectal tumors might be predictive for cetuximab efficacy and resistance. We evaluated tumor mRNA expression of the EGF ligands [*AREG*, betacellulin (*BTC*), *EGF*, epigen (*EPGN*), *EREG*, heparin binding-EGF (*HBEGF*), and tumor growth factor-α (*TGFA*)], and their receptors (*EGFR*, *HER2*, *HER3*, and *HER4*). In addition, *CD73*, *DUSP4*, and *PHLDA1* gene expression has been correlated to cetuximab resistance in several single-arm monotherapy studies of colorectal cancer (13, 19); therefore, we also evaluated their utility as prognostic and predictive markers in this study. The closure of CALGB 80203 after partial enrollment limits the power of our retrospective analysis and we wish to emphasize that conclusions should be considered preliminary until they can be verified in larger randomized studies. Although the number of patients is limited, the inclusion of *KRAS*-mutant (Mut) patients in the cetuximab arms of this study cannot be repeated in the future due to ethical concerns. Therefore, the sample population in CALGB 80203 gives us a unique opportunity to investigate pathways relevant to cetuximab response in *KRAS*-Mut patients. This is one of the first randomized studies to evaluate predictive gene expression markers of cetuximab efficacy and resistance in first-line treatment of mCRC (21).

Patients and Methods

Study design and patients

Patients with previously untreated, metastatic adenocarcinoma of the colon or rectum were randomized to FOLFIRI, FOLFIRI + cetuximab, FOLFOX, or FOLFOX + cetuximab treatment groups. This was a multicenter trial; 238 patients were randomized to treatment. Consent for biomarker analyses was optional. The protocol was approved by the Institutional Review Boards at each participating institution. This retrospective analysis conforms to the reporting guidelines established by the REMARK criteria.

Sample collection

Formalin-fixed, paraffin-embedded (FFPE) baseline tumor samples were collected during study enrollment. A total of 110 consenting patients (48%) had at least one paraffin block of primary colon or rectum tumor available for analysis. Seven samples were further excluded from this analysis due to quality and quantity issues related to the RNA isolation (Fig. 1).

KRAS mutational analysis

KRAS mutation status was determined by real-time PCR using the TheraScreen: KRAS Mutation Test Kit from Qiagen-DxS Diagnostic Innovations, which is able to detect the seven common mutations of the *KRAS* gene at codons 12 and 13 (G12A, G12D, G12R, G12C, G12S, G12V, and G13D). Analysis was performed in the Alliance molecular reference laboratory of Dr. Greg Tsongalis (Dartmouth Medical School, Hanover, NH).

RNA isolation and qRT-PCR analysis

A hematoxylin and eosin (H&E)–stained image of the tumor sample was reviewed by a pathologist to ensure the presence of >70% tumor tissue within the sample and quality of the tumor. If samples were <70% tumor, macro-dissection was performed manually. FFPE tumor biopsies were cut at the CALGB (Alliance) pathology coordinating office and shipped overnight to the Alliance molecular reference laboratory at Duke University. RNA was isolated from six 10-μm sections using the Ambion RecoverAll Total Nucleic Isolation Kit according to the manufacturer's protocol (Ambion-Life Technologies). RNA (200 ng) from each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Life Technologies). TaqMan quantitative PCR was performed for EGF-related

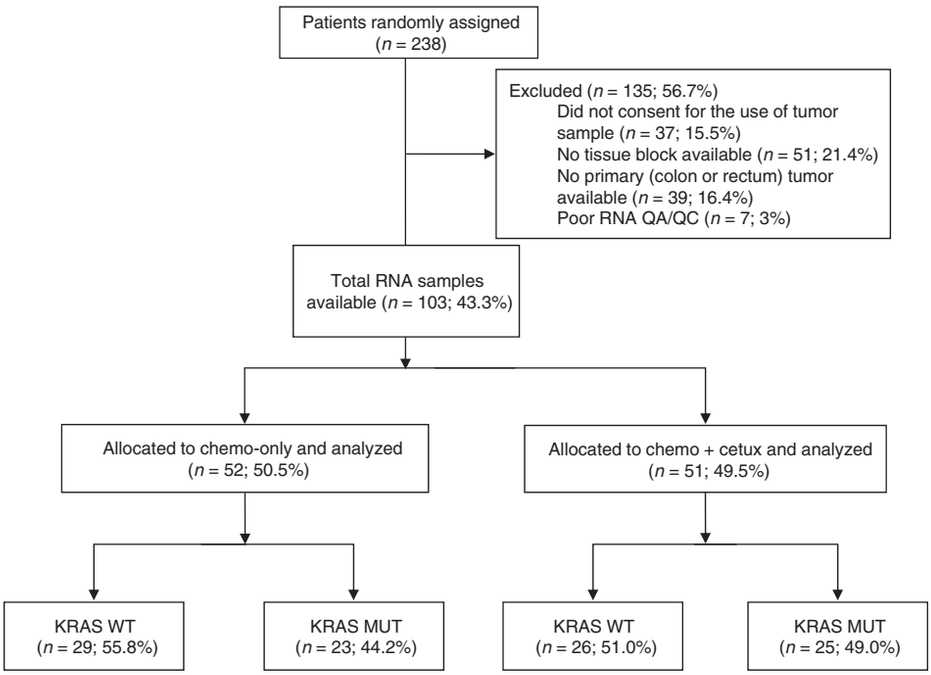

Figure 1.
Consort diagram showing patient enrollment numbers and groups.

gene expression (primer-probe sets described in Supplementary Table S1), using the ABI 7900HT Real-Time PCR System (Applied Biosystems-Life Technologies). The log-transformed relative amounts of mRNA expression were normalized to β-actin mRNA and expressed as $\log_2{}^{-(\text{CycleX-Cycle}\beta\text{-actin})} = -(\text{CycleX–Cycle}\beta\text{-actin})$, where C_T is the threshold cycle. TaqMan gene expression assays were chosen for each gene to span exon–exon junctions and have small amplicons <100 base pairs to allow for specific and sensitive detection of degraded RNA. Life Technologies TaqMan Gene Expression Assays have amplification efficiencies of approximately 100% (±10%). The β-actin endogenous control was used in this analysis. We observed uniform expression of β-actin across the mCRC tumor samples in this study. The mean C_T value was 23.6 cycles with a standard deviation of 1.9 cycles across the CALGB 80203 sample population. Duplicate samples with C_T standard deviation greater than 0.5 cycles were re-run for improved qPCR reproducibility.

Statistical analysis

Expression levels were normalized relative to β-actin, as described above, and analyzed as continuous measures. A Kendall tau analysis was performed to identify coregulated genes. Univariate Cox (22) regression was used to identify markers prognostic of clinical outcomes (OS and PFS), and the resulting *P* values, HRs, and 95% CIs are reported. To identify predictive markers, expression level was correlated with clinical outcomes (OS and PFS) using multiplicative Cox proportional hazards models to test for interaction between genetic expression and treatment (chemo vs. chemo + cetuximab). Visualizations of the resulting effect sizes are provided in the form of forest plots. The forest plots illustrate the HRs of the expression levels (and the corresponding 95% CI) within each treatment group, and the *P* values for the tests of interaction are provided. The Kaplan–Meier plots of OS and PFS were generated as additional visualizations of selected predictive markers, with separate curves for each

Table 1. Patient characteristics

	Overall whole population	Overall biomarker population	Chemo-only (biomarker population)	Chemo + cetux (biomarker population)
	n (%)	*n* (%)	*n* (%)	*n* (%)
Patients	238 (100)	103 (43)	52 (50.5)	51 (49.5)
Age, y				
Median	61.3	61.1	61.3	60.9
Range	22–84.4	22–83.3	22–83.2	40.4–83.3
Gender male	140 (58.9)	57 (55.3)	27 (51.9)	30 (58.8)
Race white	207 (87.0)	91 (88.3)	45 (86.5)	46 (90.2)
ECOG PS				
0	125 (52.5)	51 (49.5)	25 (48.1)	26 (51)
1	113 (47.5)	52 (50.5)	27 (51.9)	25 (49)
KRAS-WT	94/165 (57)	55 (53.4)	29 (55.8)	26 (51)
Median OS (95%CI)	23.0 (20.6–26.1)	26.4 (22.6–32)	22.8 (16.7–33)	27.6 (23.4–38.0)
Median PFS (95%CI)	11.05 (9.79–13.04)	9.67 (8.05–12.45)	9.66 (8.34–12.6)	10.25 (6.9–15.3)
Response rate (CR/PR)	104 (43.7)	42 (40.8)	20 (38.5)	22 (43.1)

combination of treatment group and expression level (where expression level is dichotomized at the median as "high" or "low"). Analyses were conducted using all patients, as well as separately within *KRAS*-WT and *KRAS*-Mut subgroups, due to known differential responses to cetuximab across these populations. The reported *P* values have not been adjusted for multiple testing. Because of the small sample size, uncorrected *P* values, and retrospective nature of this study, results should be considered exploratory and hypothesis-generating in nature. Further validation of predictive markers in other datasets will be necessary before they can be applied prospectively. Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center. All clinical data were locked on March 5, 2012. Statistical analyses and figures were generated using the *R* software environment for statistical computing and graphics (23) with the *survival* (22) package.

Results

Patient characteristics

Patients (238) with previously untreated mCRC were enrolled and randomly assigned to one of four treatment groups: FOLFOX, FOLFOX + cetuximab, FOLFIRI, or FOLFIRI + cetuximab. The FOLFOX and FOLFIRI treatment groups showed similar response rates, PFS and OS (20). Because of the small size of this study and similar outcomes across the FOLFOX and FOLFIRI treatment groups, these groups were combined into chemotherapy (chemo) only and chemo + cetuximab cohorts for this analysis. Patient characteristics of the biomarker population were similar to those of the overall population (Table 1). Although most studies have indicated that *KRAS* exon 2 mutations comprise approximately 40% of the colorectal cancer patient population, the biomarker population in this study had a slightly higher proportion of *KRAS*-Mut patients (Table 1). Within the biomarker population, the chemo + cetuximab cohort showed longer median PFS and OS times with higher response rates compared with the chemo-only cohort, but these differences were not statistically significant.

FFPE tissue blocks from the primary tumor site (colon or rectum) were processed from 110 patients; however, seven RNA samples were excluded because of RNA quality and quantity issues, leaving 103 patients (43%) to be included in this RNA biomarker analysis (Fig. 1). These patients were evenly distributed within the chemo-only and chemo + cetuximab treatment groups (52 vs. 51 patients). The median follow-up time for all 103 patients included in the biomarker cohort was 69.2 months.

Gene expression in primary tumors

Expression of 14 genes related to the EGF-signaling pathway (*AREG*, *BTC*, *CD73*, *DUSP4*, *EGF*, *EGFR*, *EPGN*, *EREG*, *HBEGF*, *HER2*, *HER3*, *HER4*, *PHLDA1*, and *TGFA*) was analyzed using TaqMan qRT-PCR from the primary tumors. Most genes were expressed at detectable levels in >90% patients (Supplementary Table S1). Gene expression was most strongly correlated between *EREG* and *AREG* ($\tau = 0.553$), with *HER2* and *HER3* also showing strong coexpression ($\tau = 0.475$; Supplementary Table S2). *EPGN* was coexpressed with both *HER4* ($\tau = 0.500$) and *EGF* ($\tau = 0.571$), but the low expression levels of these genes may affect interpretation of these results (Supplementary Table S1).

Prognostic gene expression biomarkers

The baseline gene expression levels were tested for association with OS and PFS using Cox proportional hazards

Table 2. Prognostic analyses of all markers for association with OS and PFS

	All patients		KRAS-WT		KRAS-Mut	
Gene	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
OS						
AREG	1.01 (0.88–1.15)	0.923	0.97 (0.82–1.16)	0.760	1.07 (0.89–1.30)	0.475
BTC	1.01 (0.85–1.21)	0.903	1.05 (0.83–1.34)	0.678	1.01 (0.75–1.35)	0.963
CD73	1.05 (0.91–1.21)	0.495	1.06 (0.88–1.27)	0.536	1.04 (0.83–1.30)	0.751
DUSP4	0.99 (0.86–1.13)	0.884	1.04 (0.89–1.23)	0.599	0.91 (0.70–1.18)	0.473
EGF	0.84 (0.68–1.03)	0.093	0.81 (0.63–1.04)	0.098	1.04 (0.61–1.76)	0.890
EGFR	1.09 (0.91–1.30)	0.372	1.04 (0.81–1.34)	0.748	1.18 (0.88–1.59)	0.272
EPGN	0.86 (0.60–1.23)	0.399	0.96 (0.58–1.59)	0.871	1.00 (0.59–1.68)	0.988
EREG	0.94 (0.86–1.03)	0.212	0.87 (0.77–0.98)	0.017	1.07 (0.91–1.25)	0.405
HBEGF	0.87 (0.73–1.04)	0.121	0.86 (0.66–1.12)	0.261	0.87 (0.68–1.11)	0.250
HER2	0.78 (0.60–1.02)	0.071	0.83 (0.61–1.14)	0.246	0.72 (0.41–1.28)	0.264
HER3	0.98 (0.81–1.18)	0.831	1.03 (0.81–1.31)	0.785	0.90 (0.64–1.28)	0.565
HER4	0.88 (0.70–1.11)	0.283	0.87 (0.64–1.19)	0.391	0.84 (0.53–1.31)	0.414
PHLDA1	1.06 (0.87–1.29)	0.567	1.06 (0.81–1.38)	0.679	1.21 (0.85–1.72)	0.299
TGFA	1.01 (0.83–1.22)	0.952	1.06 (0.85–1.32)	0.621	0.84 (0.54–1.29)	0.422
PFS						
AREG	0.91 (0.80–1.03)	0.144	0.90 (0.77–1.06)	0.220	0.93 (0.77–1.13)	0.461
BTC	0.89 (0.76–1.05)	0.172	0.94 (0.75–1.17)	0.578	0.84 (0.63–1.12)	0.234
CD73	0.99 (0.86–1.14)	0.910	1.02 (0.85–1.22)	0.866	0.97 (0.78–1.21)	0.799
DUSP4	0.95 (0.83–1.08)	0.412	0.98 (0.84–1.15)	0.799	0.89 (0.69–1.14)	0.360
EGF	0.89 (0.74–1.07)	0.223	0.86 (0.68–1.09)	0.202	1.16 (0.75–1.80)	0.492
EGFR	0.89 (0.73–1.07)	0.220	0.80 (0.58–1.09)	0.168	0.95 (0.73–1.23)	0.696
EPGN	0.96 (0.67–1.36)	0.815	1.04 (0.62–1.75)	0.890	1.01 (0.60–1.71)	0.963
EREG	0.89 (0.80–0.98)	0.016	0.84 (0.74–0.96)	0.008	0.95 (0.82–1.11)	0.526
HBEGF	0.87 (0.73–1.03)	0.117	0.92 (0.71–1.19)	0.507	0.83 (0.66–1.04)	0.103
HER2	0.64 (0.49–0.85)	0.002	0.66 (0.47–0.92)	0.013	0.65 (0.38–1.12)	0.123
HER3	0.87 (0.74–1.04)	0.127	0.91 (0.73–1.14)	0.425	0.80 (0.59–1.10)	0.174
HER4	0.80 (0.62–1.02)	0.067	0.79 (0.56–1.12)	0.180	0.77 (0.50–1.17)	0.180
PHLDA1	0.95 (0.79–1.15)	0.618	0.97 (0.76–1.24)	0.827	1.00 (0.71–1.39)	0.976
TGFA	0.90 (0.73–1.12)	0.359	0.95 (0.74–1.23)	0.704	0.81 (0.53–1.22)	0.306

regression modeling. Prognostic univariate Cox regression analyses were conducted across all patients, and within *KRAS*-WT and *KRAS*-Mut subgroups. For OS across all patients, none of the assayed genes were identified as statistically significant prognostic markers for OS across all patients (Table 2), but favorable prognostic trends were noted for *HER2* (HR, 0.78; 95% CI, 0.60–1.02; $P = 0.071$) and *EGF* (HR, 0.84; 95% CI, 0.68–1.03; $P = 0.093$). For OS, *EREG* expression was favorably prognostic for OS in the *KRAS*-WT group (HR, 0.87; 95% CI, 0.77–0.98; $P = 0.017$). For PFS, *HER2* (HR, 0.64; 95% CI, 0.49–0.85; $P = 0.002$) and *EREG* (HR, 0.89; 95% CI, 0.80–0.98; $P = 0.016$) were favorable prognostic markers across all patients. This effect seems to be driven by the *KRAS*-WT subgroup. Both *HER2* (HR, 0.66; 95% CI, 0.47–0.92; $P = 0.013$) and *EREG* (HR, 0.84; 95% CI, 0.74–0.96; $P = 0.008$) were significant prognostic markers in the *KRAS*-WT group, but failed to show significance in the *KRAS*-Mut group (*HER2*, $P = 0.123$; *EREG*, $P = 0.526$). The prognostic associations of each assayed gene with OS and PFS are included in Supplementary Figs. S1 and S2.

Predictive gene expression biomarkers

Cox proportional hazards models of OS and PFS were used to test for interaction between treatment and continuous tissue gene expression, and identified expression of *HER3* and *CD73* as potential predictive markers for benefit or lack of benefit from cetuximab. Forest plots of the HR of gene expression by treatment group are presented for OS and PFS outcomes. Markers with a $P_{interaction} \leq 0.2$ are shown in Figs. 2 and 3, while a complete analysis showing all markers is included in Supplementary Figs. S3 and S4.

Higher levels of *HER3* expression showed evidence of being predictive for lack of benefit from cetuximab, an effect that appeared restricted to the *KRAS*-WT group. For OS in the *KRAS*-WT group, the HR for chemo + cetuximab was 1.15 (95% CI, 0.81–1.62) and the HR in the chemo-only group was 0.48 (95% CI, 0.27–0.87; $P_{interaction} = 0.029$; Fig. 2A). However, in the *KRAS*-Mut population, *HER3* was not predictive of either OS or PFS benefit from cetuximab (Figs. 2B and 3B).

Gene expression of *CD73* showed a similar trend toward predicting for OS benefit from cetuximab in the *KRAS*-WT ($P_{interaction} = 0.14$) and *KRAS*-Mut ($P_{interaction} = 0.092$) groups. Higher levels of *CD73* expression predict for PFS benefit from cetuximab, an effect that appeared to be consistent in both *KRAS*-WT and *KRAS*-Mut groups. For PFS in the *KRAS*-WT group, the HR was 0.91 (95% CI, 0.70–1.18) for the chemo + cetuximab group and 1.57 (95% CI, 1.11–2.23) for the chemo-only group ($P_{interaction} = 0.026$). For PFS in the *KRAS*-Mut group, the HR was 0.80 (95% CI, 0.60–1.07) for the chemo + cetuximab and 1.29 (95% CI, 0.91–1.83) for the chemo-only group ($P_{interaction} = 0.025$). The Kaplan–Meier plots of high and low expression of *HER3* and *CD73* (dichotomized at the median) are also shown (Fig. 4).

Discussion

To date, the search for predictive markers for anti-EGFR therapies has focused largely on driver mutations, such as *KRAS*, *NRAS*, *RAF*, and *PI3K*. However, the importance of nonmutated factors in the HER axis is supported by several preclinical and clinical reports (18, 21, 24). For these reasons, we undertook an analysis of gene expression of all HER axis ligands and receptors, as well as other top candidates that had been previously identified (19).

Our analysis of CALGB 80203 is one the largest analyses of gene expression in a first-line mCRC study to date and focused exclusively on defined candidates previously reported in the literature with known biologic relevance to the EGFR axis. A key advantage of CALGB 80203 for biomarker analyses is its use of randomization between chemotherapy with and without cetuximab. Without randomization, the prognostic and predictive roles of candidate markers cannot be distinguished and their roles may be confounded or obscured. Nevertheless, our results should be considered exploratory due to the limited sample size of the study, the number of markers analyzed, and the potential for higher order interactions between markers, between markers and *KRAS* status, and with FOLFOX versus FOLFIRI treatment.

Despite these limitations, our findings suggest both the HER axis and inflammatory pathways mediate resistance to cetuximab.

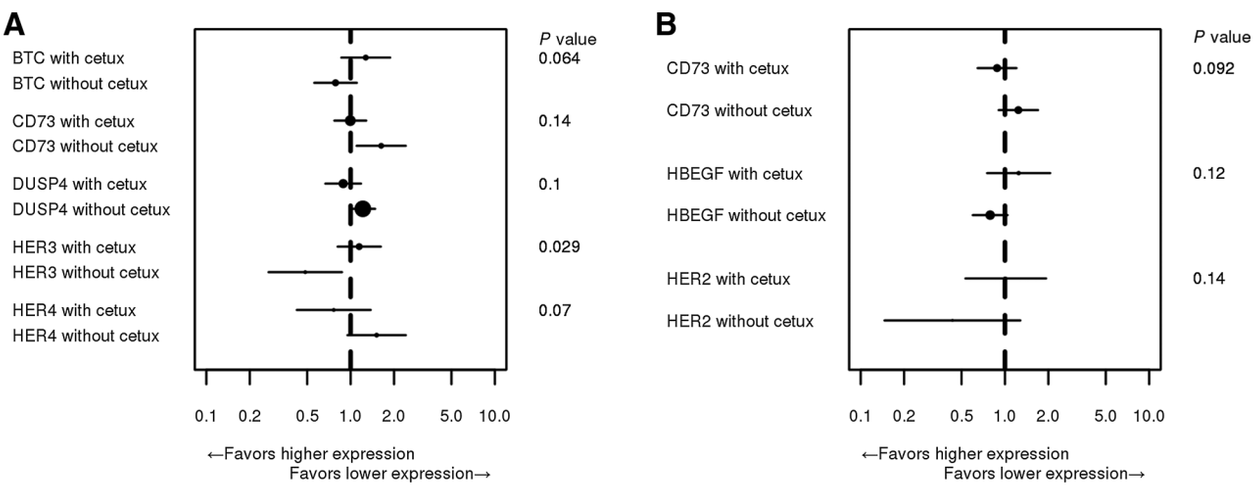

Figure 2.
Forest plots showing the associations of gene expression and treatment group with OS in *KRAS*-WT (A) and *KRAS*-Mut (B) patients. Only genes with $P_{interaction} \leq 0.2$ are shown. The length of the line indicates the 95% CI, and the diameter of the median dot is inversely proportional to the standard deviation.

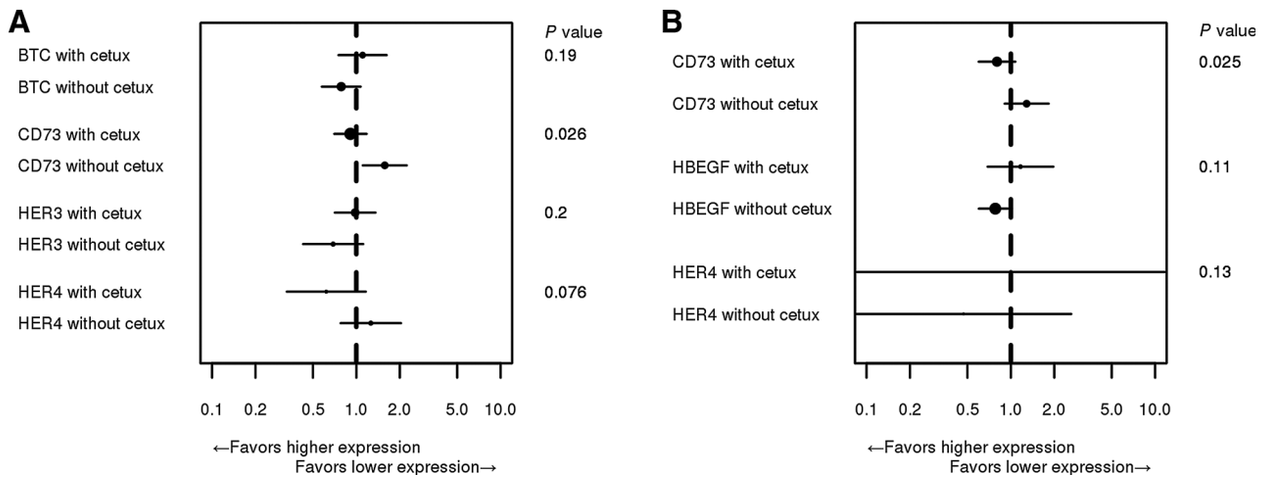

Figure 3.

Forest plots showing the associations of gene expression and treatment group with PFS in *KRAS*-WT (A) and *KRAS*-Mut (B) patients. Only genes with $P_{interaction} \leq 0.2$ are shown. The length of the line indicates the 95% CI, and the diameter of the median dot is inversely proportional to the standard deviation.

High *HER3* levels were associated with both resistance and lack of benefit from cetuximab. This effect was most prominent in patients whose tumors were *KRAS*-WT. Expression of other markers in the HER axis showed a trend for predicting benefit from cetuximab. Her3 is kinase-deficient, but it heterodimerizes with Her2 to generate a potent signaling module. Her3 contains SH2 domains that, when phosphorylated by coreceptors, can activate the downstream PI3K pathway (25). Her2 and Her3 are also activated by EGF and BTC providing additional means for these ligands to support cell signaling and growth (26). Although signaling through Her3 has been shown to confer resistance to anti-EGFR agents in preclinical models (27, 28), evidence for this effect in colorectal cancer patients treated with cetuximab has been retrospective and lacked the randomization of this study (29, 30). The coexpression of *AREG* and *EREG* has been shown to play a role in the physiologic response to cetuximab treatment (18). The coexpression of *HER2* and *HER3* has also been shown previously (31) and is of particular interest as Her3 is capable of activating downstream pathways even within the context of Her2 inhibition (27). Our data provide additional clinical rationale for the evaluation of strategies that cotarget EGFR and Her3 in patients with mCRC.

We also identified tissue *CD73* expression as a potential predictive marker for benefit from cetuximab. *CD73* was among the top markers that correlated with outcome in the report by Baker and colleagues (19). Intriguingly, our results were consistent in both *KRAS*-WT and *KRAS*-Mut populations. CD73 is known to play a central role in regulating multiple inflammatory responses, primarily by controlling levels of extracellular adenosine (32). *CD73* is regulated by multiple factors, including RAS, STAT, HIF, and TGFβ (33, 34). The biology of CD73 and adenosine signaling has been extensively reviewed (35–37). CD73 acts with CD39 to convert inflammatory extracellular ATP to anti-inflammatory adenosine. CD73 is expressed on endothelial cells and Tregs and its overexpression may impair the ability of the immune system to respond to growing malignancies (35–37). The recent success of immune-activating agents targeting PD-1/PD-L1 is an interesting analogy showing the potential benefit of upregulating the immune system to aid tumor inhibition. Several preclinical studies have provided support for targeting CD73 as a therapeutic mechanism. In Fig. 4B, patients in the high CD73 group who received chemotherapy only seemed to have the shortest OS indicating that larger studies may potentially identify a negative prognostic effect of high CD73 expression.

Multiple preclinical models have shown that inflammatory factors, including IL6, IL8, STAT3, and TGFβ, may mediate resistance to anti-EGFR therapies. Intriguingly, cetuximab induces an acneiform rash, which is predominantly neutrophillic and responds to anti-inflammatory agents, such as minocycline and steroids (38, 39). Whether similar infiltrates are also induced in the tumor microenvironment in patients is not known but is highly plausible. In this context, our *CD73* data suggest potentially important roles for inflammation and immunity as mechanisms of sensitivity and resistance to cetuximab treatment. The predictive role for *CD73* in patients whose tumors are *KRAS*-Mut suggests that inflammation could be an additional mechanism of *RAS*-mediated resistance to cetuximab. Many immune subpopulations, particularly those mediating inflammation, can exert a negative effect on antitumor immunity, including cell types regulated by CD73 such as neutrophils, Tie2-expressing macrophages, and Tregs (40–45).

Macrodissection enriched the tumor content for each sample, but this still represents a complex mixture of both tumor and the surrounding stroma. The expression patterns we have observed may not be intrinsic to the tumor only. In fact, these samples represent a baseline snapshot of expression that may reflect complex signaling between the tumor and its environment. Further studies are required to evaluate changes in mRNA expression associated with either cetuximab treatment or the progression of the disease.

Our results serve to extend and refine many of the findings initially reported by Baker and colleagues (19). However, our results also differ somewhat from the results of those studies. These differences may relate to numerous factors, including potential differences in study populations, preanalytic considerations, which analytes were measured, and whether the studies were or were not randomized. A major strength of the current analysis is the randomization used in the parent study. In a recent

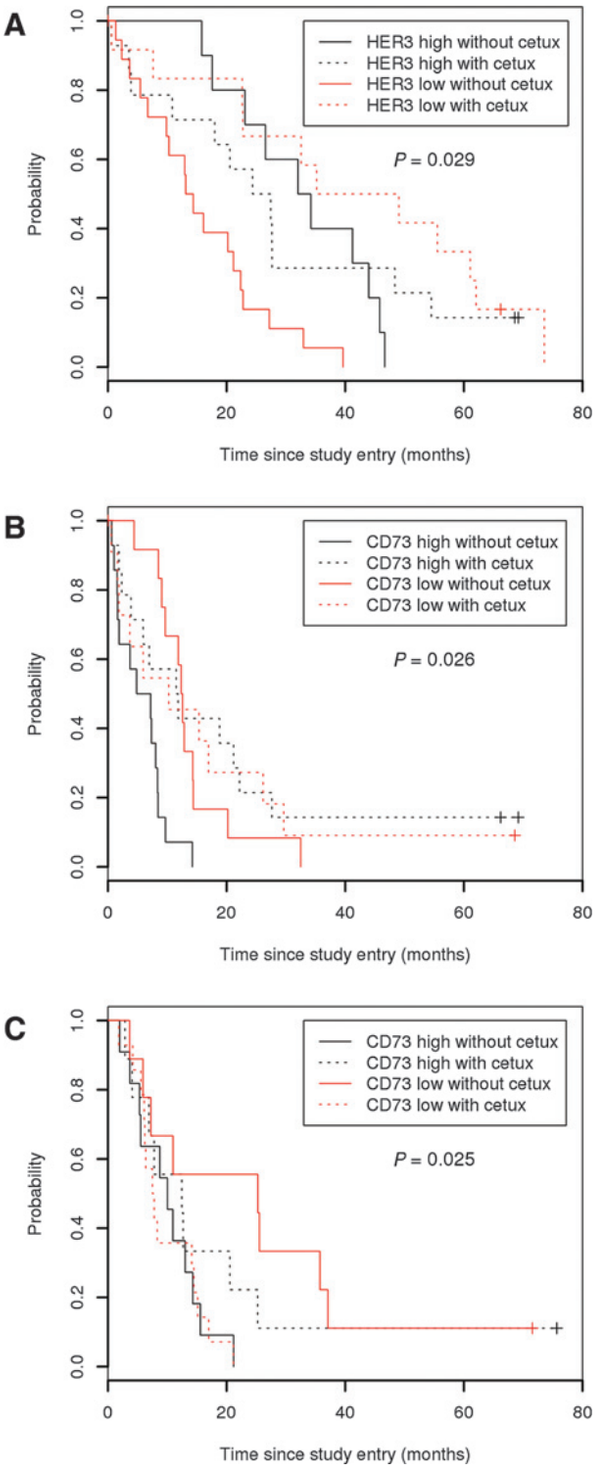

Figure 4.
The Kaplan–Meier plots of tumor gene expression levels significantly associated with outcome. OS by HER3 expression in KRAS-WT pts (A), PFS by CD73 expression in KRAS-WT pts (B), PFS by CD73 expression in KRAS-Mut pts (C; all groups dichotomized at the median). $P_{interaction}$ values are shown.

report from the randomized CO.17 study of cetuximab versus best supportive care in patients with refractory colorectal cancer, the combination of *KRAS* status plus *EREG* was found to be a significant predictor of benefit from cetuximab, although *EREG* alone was of only borderline significance (21). Other candidate markers beyond *EREG* were not reported in that analysis.

In conclusion, using samples from the randomized CALGB 80203 study in first-line mCRC, we identified two strong potential candidate predictors of benefit from cetuximab, *HER3* and *CD73*. These data implicate specific and targetable factors in the HER axis and inflammation as key mediators of resistance to cetuximab.

Disclosure of Potential Conflicts of Interest

S.M. Cushman is an employee of Novartis Pharmaceutical Corporation. A.P. Venook is a consultant/advisory board member for Merck Serono. H.I. Hurwitz reports receiving commercial grants from Bristol-Myers Squibb, Genentech/Roche, and Lilly, and is a consultant/advisory board member for Bristol-Myers Squibb, Genentech/Roche, Incyte, Lilly, Regeneron, and Sanofi. A.B. Nixon reports receiving commercial grants from Amgen, Pfizer, and Tracon Pharmaceuticals, and is a consultant/advisory board member for GlaxoSmithKline and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors gratefully acknowledge the invaluable contributions of the patients, their families, and the all the investigators who participated in this trial. The authors give special recognition to Dr. Greg Tsongalis (Dartmouth Medical School) for providing the *KRAS* mutational data.

The following institutions participated in this study: Cancer Centers of the Carolinas, Greenville, South Carolina, Jeffrey K. Giguere, supported by CA29165; Dana-Farber Cancer Institute, Boston, Massachusetts, Harold J. Burstein, supported by CA32291; Dartmouth Medical School-Norris Cotton Cancer Center, Lebanon, New Hampshire, Konstantin Dragnev, supported by CA04326; Georgetown University Medical Center, Washington, DC, Bruce Cheson, supported by CA77597; Grand Rapids Clinical Oncology Program, Grand Rapids, Michigan, Martin J. Bury; Hematology-Oncology Associates of CNY CCOP, Syracuse, NY, Jeffrey Kirshner, M.D., supported by CA45389; Kansas City Community Clinical Oncology Program CCOP, Kansas City, Missouri, Rakesh Gaur; Mount Sinai Medical Center, Miami, Florida, Michael A. Schwartz, supported by CA45564; Missouri Baptist Medical Center, St. Louis, Missouri, Alan P. Lyss; Missouri Valley Consortium-CCOP, Omaha, Nebraska, Gamini S. Soori; Northern Indiana Cancer Research Consortium CCOP, South Bend, Indiana, Rafat Ansari, supported by CA86726; Rhode Island Hospital, Providence, Rhode Island, William Sikov, supported by CA08025; Roswell Park Cancer Institute, Buffalo, New York, Ellis Levine, supported by CA59518; Southeast Cancer Control Consortium Inc. CCOP, Goldsboro, North Carolina, James N. Atkins, supported by CA45808; State University of New York Upstate Medical University, Syracuse, New York, Stephen L. Graziano, supported by CA21060; University of Chicago, Chicago, Illinois, Hedy L. Kindler, supported by CA41287; University of Illinois MBCCOP, Chicago, Illinois, David J. Peace, supported by

CA74811; University of Iowa, Iowa City, Iowa, Daniel A. Vaena, supported by CA47642; University of Massachusetts Medical School, Worcester, MA, William V. Walsh, supported by CA37135; University of Minnesota, Minneapolis, Minnesota, Bruce A. Peterson, supported by CA16450; University of Nebraska Medical Center, Omaha, Nebraska, Apar Ganti, supported by CA77298; University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, Thomas C. Shea, supported by CA47559; Wake Forest University School of Medicine, Winston-Salem, North Carolina, David D. Hurd, supported by CA03927; Walter Reed Army Medical Center, Washington, DC, David C. Van Echo, supported by CA26806; Washington University School of Medicine, St. Louis, Missouri, Nancy Bartlett, supported by CA77440; Weill Medical College of Cornell University, New York, New York, John Leonard, supported by CA07968.

Grant Support

Research reported in this publication was supported by the National Cancer Institute of the NIH under award numbers U10CA180821, U10CA180882, CA31946, CA33601, CA47577, and CA60138.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 10, 2014; revised November 4, 2014; accepted December 4, 2014; published OnlineFirst December 17, 2014.

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