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

Stephanie M. Cushman, Chen Jiang, Ace J. Hatch, Ivo Sheterve, Alexander B. Sibley, Donna Niedzwiecki, Alan P. Venook, Kouros Owzar, Herbert I. Hurwitz, and Andrew B. Nixon

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), NTSE (CD73), DUSP4, EGF, EGRF, epigen (EPGN), epiregulin (EREG), HBEGF, ERBB2 (HER2), ERBB3 (HER3), ERBB4 (HER4), PHLD1A, and TGF. 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; Pinteraction = 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; Pinteraction = 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/HPR 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...
other patients. 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.

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, 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 Recover-All 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 genes 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 epieregulin (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 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 EGFR signaling–related genes in colorectal tumors might be predictive for cetuximab efficacy and resistance. We evaluated tumor mRNA expression of the EGFR ligands [AREG, betacellulin (BTC), EGF, epiregulin (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).
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 \( b\)-actin mRNA and expressed as log 2
\[ \text{log}_2 \left( \frac{C_T}{CT_{\text{b-actin}}} \right) = \text{cycle}_{\text{b-actin}} - \text{cycle}_{\text{mRNA}} \]
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% (\( \leq 10\% \)). The \( b\)-actin endogenous control was used in this analysis. We observed uniform expression of \( b\)-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 \( b\)-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

<table>
<thead>
<tr>
<th></th>
<th>Overall whole population</th>
<th>Overall biomarker population</th>
<th>Chemo-only (biomarker population)</th>
<th>Chemo + cetux (biomarker population)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Age, y</td>
<td>238 (100)</td>
<td>103 (43)</td>
<td>52 (50.5)</td>
<td>51 (49.5)</td>
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<tr>
<td>Race white</td>
<td>207 (87.0)</td>
<td>91 (88.3)</td>
<td>45 (86.5)</td>
<td>46 (90.2)</td>
</tr>
<tr>
<td>Gender male</td>
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<td>57 (55.3)</td>
<td>27 (51.9)</td>
<td>30 (58.8)</td>
</tr>
<tr>
<td>Race white</td>
<td>207 (87.0)</td>
<td>91 (88.3)</td>
<td>45 (86.5)</td>
<td>46 (90.2)</td>
</tr>
<tr>
<td>ECOG PS</td>
<td>0</td>
<td>125 (52.5)</td>
<td>25 (48.1)</td>
<td>26 (51)</td>
</tr>
<tr>
<td>Median OS (95%CI)</td>
<td>23.0 (20.6–26.1)</td>
<td>26.4 (22.6–32)</td>
<td>22.8 (16.7–33)</td>
<td>27.6 (23.4–38.0)</td>
</tr>
<tr>
<td>Median PFS (95%CI)</td>
<td>11.05 (9.79–13.04)</td>
<td>9.67 (8.05–12.45)</td>
<td>9.66 (8.34–12.6)</td>
<td>10.25 (6.9–15.3)</td>
</tr>
<tr>
<td>Response rate (CR/PR)</td>
<td>104 (43.7)</td>
<td>42 (40.8)</td>
<td>20 (38.5)</td>
<td>22 (43.1)</td>
</tr>
</tbody>
</table>

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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 TGFα) 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 (r = 0.553), with HER2 and HER3 also showing strong coexpression (r = 0.475; Supplementary Table S2). EPGN was coexpressed with both HER4 (r = 0.500) and EGF (r = 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 analysis.
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 were presented for OS and PFS outcomes. Markers with a \( P_{\text{interaction}} \leq 0.2 \) 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; \( P_{\text{interaction}} = 0.029 \); 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 (\( P_{\text{interaction}} = 0.14 \)) and KRAS-Mut (\( P_{\text{interaction}} = 0.092 \)) 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 (\( P_{\text{interaction}} = 0.026 \)). 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 (\( P_{\text{interaction}} = 0.025 \)). 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 of 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 FOLFIRI versus FOLFOX treatment.

Despite these limitations, our findings suggest both the HER axis and inflammatory pathways mediate resistance to cetuximab.
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. HERO contains SH2 domains that, when phosphorylated by coreceptors, can activate the downstream PI3K pathway (25). Her2 and HERO are also activated by EGF and BTC providing additional means for these ligands to support cell signaling and growth (26). Although signaling through HERO 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 HERO has also been shown previously (31) and is of particular interest as HERO 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 HERO in patients whose tumors were or were not randomized. A major strength of the current studies has been the randomization used in the parent study. In a recent analysis is the randomization used in the parent study. In a recent

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.

Predictive Biomarkers for Cetuximab in Colorectal Cancer
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 Tacon Pharmaceuticals, and is a consultant/advisory board member for GlaxoSmithKline and Novartis. No potential conflicts of interest were disclosed by the other authors.

**Disclaimer**

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Cushman, H.I. Hurwitz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Cushman, C. Jiang, A.J. Hatch, I. Shterev, A.B. Sibley, A.P. Venook, K. Owzar, H.I. Hurwitz, A.B. Nixon

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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* values are shown.

The following content is only for clarification and does not change the original text:

**Clinical Cancer Research**

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**Conflict of Interest**

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