Plasma Protein Biomarkers in Advanced or Metastatic Colorectal Cancer Patients Receiving Chemotherapy With Bevacizumab or Cetuximab: Results from CALGB 80405 (Alliance)

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ABSTRACT

Purpose: CALGB 80405 compared the combination of first-line chemotherapy with cetuximab or bevacizumab in the treatment of advanced or metastatic colorectal cancer (mCRC). Although similar clinical outcomes were observed in the cetuximab-chemotherapy group and the bevacizumab-chemotherapy group, biomarkers could identify patients deriving more benefit from either biologic agent.

Patients and Methods: In this exploratory analysis, the Angiome, a panel of 24 soluble protein biomarkers were measured in baseline plasma samples in CALGB 80405. Prognostic biomarkers were determined using univariate Cox proportional hazards models. Predictive biomarkers were identified using multivariable Cox regression models including interaction between biomarker level and treatment.

Results: In the total population, high plasma levels of Ang-2, CD73, HGF, ICAM-1, IL6, OPN, TIMP-1, TSP-2, VCAM-1, and VEGF-R3 were identified as prognostic of worse progression-free survival (PFS) and overall survival (OS). PlGF was identified as predictive of lack of PFS benefit from bevacizumab [bevacizumab HR, 1.51; 95% confidence interval (CI), 1.10–2.06; cetuximab HR, 0.94; 95% CI, 0.71–1.25; $P_{\text{interaction}} = 0.0298$] in the combined FOLFIRI/FOLFOX regimens. High levels of VEGF-D were predictive of lack of PFS benefit from bevacizumab in patients receiving FOLFOX regimen only (FOLFOX/bevacizumab HR, 1.70; 95% CI, 1.19–2.42; FOLFOX/cetuximab HR, 0.92; 95% CI, 0.68–1.24; $P_{\text{interaction}} = 0.0097$).

Conclusions: In this exploratory, hypothesis-generating analysis, the Angiome identified multiple prognostic biomarkers and two potential predictive biomarkers for patients with mCRC enrolled in CALGB 80405. PlGF and VEGF-D predicted lack of benefit from bevacizumab in a chemo-dependent manner.

See related commentaries by Mishkin and Kohn, p. 2722 and *George and Bertagnolli, p. 2725*

Introduction

Colorectal cancer is a leading cause of cancer mortality in the United States. Significant progress has been made in the past 20 years in both detection and treatment of colorectal cancer (1). In 1990s, fluorouracil became the only cytotoxic drug in colorectal cancer as continuous infusion of fluorouracil improved the overall survival (OS) from 12 to 15 months (2). Since then, combination therapies of fluorouracil with leucovorin and either irinotecan [FOLFIRI regimen (3)] or oxaliplatin [FOLFOX regimen (4)] became the first-line chemotherapeutic treatment for colorectal cancer. In 2000s, targeted therapies were added to chemotherapy, including mAbs targeting EGFR and VEGF (5, 6).

Two anti-EGFR antibodies, panitumumab and cetuximab, have been approved by FDA to treat patients with metastatic CRC (mCRC) as monotherapies and combined with chemotherapy (7, 8). EGFR binding to its ligands results in the activation of the mitogenic MAPK/ERK signaling cascade via *RAS* GTPases (9). Patients whose tumors harbor activating *RAS* mutations do not respond to EGFR-targeting agents (10–12). Current guidelines recommend the use of panitumumab and cetuximab only for patients with CRC with wild-type *RAS* (13).

There are currently four FDA approved biological agents for targeting the VEGF-pathway in patients with colorectal cancer: first-line bevacizumab which binds VEGF-A (5); second-line ramucirumab which binds VEGFR-2 (14), and ziv-aflibercept, which binds VEGFs and PlGF (15); and third-line regorafenib which blocks activity of all VEGF receptors and other tyrosine kinases (16, 17). Extensive effort has been made to identify biomarkers to guide anti-VEGF drugs, including markers at the molecular, cellular, and tissue levels (18).

Circulating protein biomarkers have gained increasing attention given the advantages of ease to obtain, cost-effectiveness, and amenability to repeated sampling. We have developed and optimized the Angiome, a panel of circulating protein biomarkers and analyzed their predictive potential in multiple trials involving thousands of patients

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Translational Relevance

CALGB 80405 was a randomized phase III trial in patients with advanced or metastatic colorectal cancer (mCRC) testing whether the addition of cetuximab or bevacizumab to the combination of either leucovorin, fluorouracil, and irinotecan (FOLFIRI) or the combination of leucovorin, fluorouracil, and oxaliplatin (FOLFOX) regimen was superior as first-line therapy in this setting. The results of the trial were negative, similar overall survival (OS) and progression-free survival (PFS) were observed for the cetuximab-chemotherapy group and the bevacizumab-chemotherapy group. In this report, we conducted an exploratory, hypothesis-generating, retrospective analysis evaluating a panel of angiogenic and inflammatory biomarkers using baseline plasma samples collected from patients enrolled in CALGB 80405 and identified PlGF and VEGF-D as potential predictive biomarkers. High PlGF levels predict lack of PFS benefit from bevacizumab independent of the chemotherapeutic regimen, whereas low VEGF-D levels predict PFS benefit from bevacizumab in patients receiving FOLFOX. Notably, the predictive potential of VEGF-D for anti-angiogenic agents has been previously reported by several groups. Given the lack of a true control arm, the findings are exploratory and hypothesis-generating in nature.

in different disease settings (19–22). Ang-2, SDF-1, and VEGF-D plasma levels were identified as predictive biomarkers for bevacizumab in patients with pancreatic cancer treated with gemcitabine ± bevacizumab in a study conducted by Cancer and Leukemia Group B (CALGB), now part of the Alliance for Clinical Trials in Oncology cooperative group, CALGB 80303 (20). Benefit from bevacizumab was associated with high levels of Ang-2 and SDF-1 and low levels of VEGF-D. The VEGF-D effect was strongest in patients in the lowest quartile of plasma VEGF-D (20). Consistently, negative predictive effect of VEGF-D has been reported in patients with mCRC treated in the Australian Gastro-Intestinal Trials Group testing Mitomycin, Avastin, and Xeloda (AGITG MAX trial; ref. 23). Despite a number of promising candidates including VEGF-D, currently there are no validated biomarkers to guide the applications of anti-angiogenic agents in any disease settings (24).

CALGB and the Southwest Oncology Group (SWOG), conducted the intergroup study, CALGB/SWOG 80405, to better guide clinical decision making for patients with advanced or mCRC (25). CALGB 80405 was initially designed to compare bevacizumab, cetuximab, and the combination of both agents in addition to FOLFIRI or FOLFOX chemotherapy regimen chosen by the physician and patient. Early in the course of the study, the combination regimen was discontinued due to results from the CAIRO2 and PACCE studies showing increased toxicity and a lack of increased survival benefit in the arms with dual VEGF and EGFR inhibition (26, 27). CALGB80405 was negative for overall survival (OS), the primary endpoint for this study. The clinical results of CALGB 80405 showed similar progression-free survival (PFS) and OS benefits from either bevacizumab-chemotherapy or cetuximab-chemotherapy groups (25).

To improve outcomes in future clinical trials of bevacizumab in mCRC, a homogenous selected patient population would be pivotal. Disease parameters and circulating biomarkers would greatly facilitate patient selection to enrich patient deriving the most benefit from anti-angiogenic drugs. As stated earlier, our Angiome analysis has successfully identified prognostic and predictive biomarkers for anti-angiogenic agents, bevacizumab included. Although prognostic biomarkers reflect how well a patient perform, independent of treatments; predictive biomarkers have the potential to identify patients benefitting the most from a specific treatment.

In this study, we assessed the Angiome expression in plasma samples collected at baseline in the primary cohort of CALGB 80405. These patients habored *KRAS* wild-type tumors, received either bevacizumab or cetuximab in addition to FOLFIRI or FOLFOX. Angiome analysis provided a snapshot of tumor angiogenesis, inflammation, and immune status at baseline. Potential prognostic and predictive biomarkers were identified.

Patients and Methods

Study design and patients

The study design of CALGB 80405 and clinical results have been reported (25) and detailed information can be found at ClinicalTrials.gov (NCT00265850). Briefly, patients with locally advanced or mCRC were randomized to receive either bevacizumab, cetuximab, or the combination of both in conjunction with chemotherapy (FOLFIRI or FOLFOX) at the discretion of the treating physician. The study was later amended to stop enrollment in the combination arm and to implement *KRAS* mutation testing. The samples used in this study were collected at baseline from the primary cohort of CALGB 80405. The primary cohort consists of 1137 *KRAS* wild-type patients randomized to the bevacizumab + chemotherapy or cetuximab + chemotherapy treatment groups. This correlative study of CALGB 80405 was approved by the Institutional Review Board at all participating centers, adhered to Guidelines for Good Clinical Practice and guiding principles laid out in the Declaration of Helsinki. Written informed consent was obtained from patients who opted to participate in this analysis.

Sample collection

Peripheral venous blood was collected at baseline from consenting patients into vacutainers containing EDTA anticoagulant. Samples were centrifuged at 2,500 *g* for 15 minutes within 30 minutes of collection. The resulting plasma was spun again at 2,500 *g* for 15 minutes. Double-spun, platelet-poor plasma was aliquoted, frozen, and shipped on dry ice to the Alliance Pathology Coordinating Office for centralized storage. Prior to analyses, samples were shipped to Duke Phase I Biomarker Laboratory, thawed once on ice, re-aliquoted, and stored at −80°C until assayed.

Protein analysis

Levels of 24 soluble protein biomarkers were measured in duplicates with the personnel blinded to the clinical results. Plasma samples from patients in the primary cohort were analyzed using the multiplex ELISA techniques on the Quanterix and Meso Scale Discovery platforms as described previously (20, 28). The analysis conforms to the guidelines established by the REMARK criteria.

Statistical considerations

All biomarker levels were log-transformed prior to analysis, to de-emphasize outliers and better approximate a normal distribution. All analyses were performed using baseline data from patients in the primary cohort with continuous values for the protein analytes. Biomarkers prognostic of clinical outcome [overall survival (OS) or progression-free survival (PFS)] were determined using univariate Cox proportional hazards models independent of treatment arm, and the resulting HR, 95% confidence intervals (CI), and asymptotic *P* values

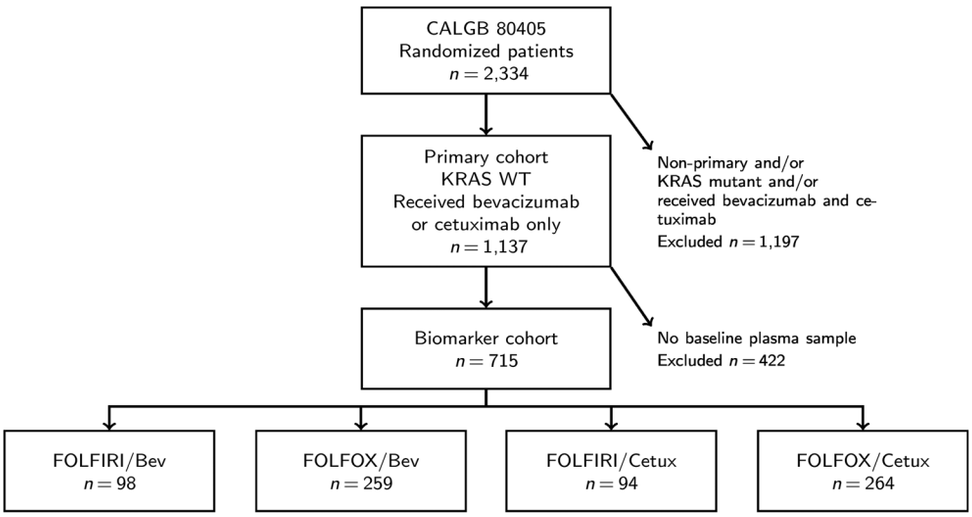

Figure 1.
Patient CONSORT diagram. Bev, bevacizumab; Cetux, cetuximab.

based on the score test were reported. Multivariable Cox regression models were used to test for interactions between biomarker levels and treatment arms to identify biomarkers predictive of benefit from bevacizumab or cetuximab and asymptotic *P* values based on the Wald test for the interaction terms were reported. All analyses were stratified by type of chemotherapy (FOLFIRI or FOLFOX) to control for any differences in baseline survival rate among these two groups. Biomarker levels were also tested for interaction with treatment arm separately within each chemotherapy subpopulation. The reported *P* values were not adjusted for multiple testing.

Table 1. Distribution of the biomarker subpopulation by treatment.

	Chemotherapy Target therapy	**FOLFIRI/ Bev**	**FOLFOX/ Bev**	**FOLFIRI/ Cetux**	**FOLFOX/ Cetux**	**Primary cohort**
	N	98	259	94	264	1,137
Age	Median	61	58	60	59	59
	(range)	(34–82)	(22–84)	(36–83)	(23–84)	(20–89)
Race	African American	9 (9.2%)	22 (8.5%)	10 (10.6%)	31 (11.7%)	129 (11.3%)
	Asian	4 (4.1%)	4 (1.5%)	2 (2.1%)	9 (3.4%)	35 (3.1%)
	Caucasian	84 (85.7%)	228 (88.0%)	81 (86.2%)	218 (82.6%)	934 (82.1%)
	Multiple	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.4%)	5 (0.4%)
	Other	1 (1.0%)	5 (1.9%)	1 (1.1%)	5 (1.9%)	34 (3.0%)
Sex	Female	37 (37.8%)	95 (36.7%)	37 (39.4%)	103 (39.0%)	440 (38.7%)
	Male	61 (62.2%)	164 (63.3%)	57 (60.6%)	161 (61.0%)	697 (61.3%)
Median	OS	35.1	30.3	31.9	28.9	29.4
outcome	(95% CI)	(29.4–41.3)	(27.1–33.2)	(26.8–40.2)	(25.3–34.6)	(27.6–31.4)
(months)	PFS	12.4	10.8	10.9	10	10.6
	(95% CI)	(10.4–14.0)	(9.5–11.7)	(9.2–14.4)	(9.2–11.3)	(9.8–11.1)
ECOG	0	60 (61.2%)	153 (59.1%)	55 (58.5%)	162 (61.4%)	657 (57.8%)
N (%)	1	38 (38.8%)	105 (40.5%)	39 (41.5%)	102 (38.6%)	478 (42.0%)
	2		1 (0.4%)			2 (0.2%)
Tumor	Metachronous	36 (36.7%)	33 (12.7%)	34 (36.2%)	45 (17.1%)	236 (20.8%)
biology	Synchronous	62 (63.3%)	225 (86.9%)	57 (60.6%)	217 (82.2%)	892 (78.5%)
	unknown		1 (0.4%)	3 (3.2%)	2 (0.8%)	9 (0.8%)
CRC	Left	63 (64.3%)	147 (56.8%)	63 (67.0%)	164 (62.1%)	689 (60.6%)
tumor	Right	24 (24.5%)	69 (26.6%)	16 (17.0%)	61 (23.1%)	280 (24.6%)
location	Transverse	3 (3.1%)	18 (7.0%)	7 (7.5%)	17 (6.4%)	62 (5.5%)
N (%)	Multiple				1 (0.4%)	1 (0.1%)
	unknown	8 (8.2%)	25 (9.7%)	8 (8.5%)	21 (8.0%)	105 (9.2%)
Primary tumor	No	76 (77.6%)	192 (74.1%)	75 (79.8%)	196 (74.2%)	845 (74.3%)
unresected at study entry	Yes	22 (22.4%)	67 (25.9%)	19 (20.2%)	68 (25.8%)	292 (25.7%)

Note: Metachronous indicates metastasis subsequent to diagnosis of primary tumor; synchronous indicates metastasis present at diagnosis of primary tumor. Values from the primary cohort are as reported in Venook and colleagues (25).
Abbreviations: Bev, bevacizumab; Cetux, cetuximab.

Hierarchical agglomerative clustering, using Euclidean distance and complete linkage, was used to identify groupings among protein biomarkers based on their baseline levels. The results were illustrated using a dendrogram. Forest plots were created as illustrations of prognostic effect sizes (HRs and corresponding 95% CIs). For the purposes of illustrating interaction effects, biomarker levels were dichotomized as "high" or "low" relative to the median, and Kaplan–Meier plots of OS and PFS were created, with separate curves for each combination of treatment group and dichotomized biomarker level. It has previously been shown that low levels of VEGF-D (first quartile) were predictive of benefit from bevacizumab in advanced pancreatic cancer and colon cancer, respectively (20, 23), so we also considered VEGF-D levels separated by quartiles. Unless otherwise stated, the results reported pertain to the analysis of biomarkers as continuous measures.

The Alliance Statistics and Data Center conducted data collection and statistical analyses on the basis of clinical data locked as of December 15, 2015. The R software environment for statistical computing and graphics and the survival extension package were used to conduct the statistical analyses and to generate the figures (29, 30). The analyses were carried out with adherence to the principles of reproducible analysis using the knitr package (31) for generation of dynamic reports and GitLab for source code management.

Data availability statement

The code for replicating the statistical analysis has been made accessible through a public source code repository (https://gitlab.oit.duke.edu/dcibioinformatics/pubs/calgb-80405-plasma).

De-identified patient data may be requested from Alliance for Clinical Trials in Oncology via concepts@alliancenctn.org if data are not publicly available. A formal review process includes verifying the availability of data, conducting a review of any existing agreements that may have implications for the project, and ensuring that any transfer is in compliance with the IRB. The investigator will be required to sign a data release form prior to transfer.

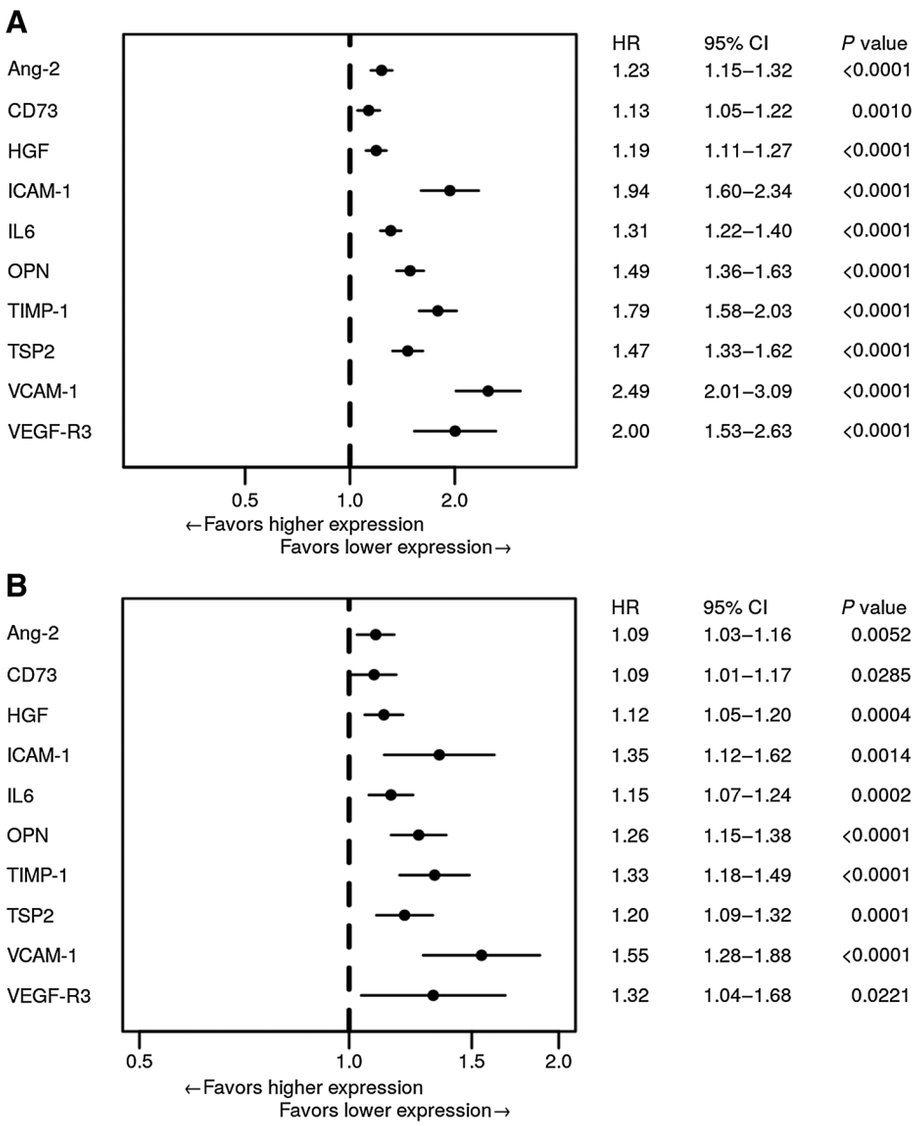

Figure 2.
Prognostic biomarkers. Forest plots of HRs illustrate the prognostic association of biomarkers for both OS (**A**) and PFS **B**) in the overall population. All 10 were negative prognostic, indicating that higher biomarker levels are associated with higher HR or favor lower expression.

Results

Patient characteristics

In CALGB 80405 primary cohort, patients with mCRC with *KRAS* wild type were randomized to receive bevacizumab (BEV) or cetuximab (Cetux), in addition to either FOLFIRI or FOLFOX as chemotherapeutic agents per physician choice. The last date of follow up was December 15, 2015, and the median follow up for surviving patients was 47.4 months (25). The demographic and survival outcomes for the total primary cohort population (n = 1,137) and the biomarker population (n = 715) are shown in **Fig. 1** and Supplementary Table S1. No significant differences were noticed regarding age, race, gender, or clinical outcomes between the biomarker population and the total primary cohort. To better present the four patient cohorts receiving different treatments, a detailed demographic table was generated. As reported in the clinical paper, there is no statistically significant difference in OS and PFS in the bevacizumab-chemotherapy group and the cetuximab-chemotherapy group (25). All four patient cohorts showed similar baseline characteristics (**Table 1**).

Biomarker assessment

A total of 24 circulating protein biomarkers were assessed using baseline plasma samples for 715 patients with mCRC enrolled in CALGB 80405. The median levels and ranges for all biomarkers are shown in Supplementary Table S2. A dendrogram illustrating the associations among these 24 biomarkers is shown in Supplementary Fig. S1. As observed in previous analyses, we noted several consistent relationships among the protein biomarkers. PlGF was tightly associated with general angiogenic biomarkers such as Ang-2 and TSP-2, whereas VEGF-A, -C, -D were more clustered with factors in TGFβ signaling pathway. CD73 and HER-3, two previously identified biomarkers predictive of benefit from cetuximab (28), were tightly clustered with each other.

Prognostic analyses

The clinical report showed similar OS and PFS across patients in all treatment groups, therefore, prognostic analysis was conducted with all patients combined. We identified ten biomarkers (Ang-2, CD73, HGF, ICAM-1, IL6, OPN, TIMP-1, TSP-2, VCAM-1, and VEGFR-3) to be prognostic for both OS and PFS in the overall population, as illustrated by the Forrest plots (**Fig. 2**). All of the biomarkers identified were negative prognostic biomarkers, that is, higher expression levels were associated with shorter times to event (OS or PFS). The strongest prognostic effects with respect to HR for OS and PFS were observed for VCAM-1 (for OS, HR, 2.49; 95% CI, 2.01–3.09; $P < 0.0001$; for PFS, HR, 1.55; 95% CI, 1.28–1.88; $P < 0.0001$). A complete list of the prognostic value for all 24 biomarkers for OS and PFS was shown in Supplementary Tables S3 and S4, respectively. All biomarkers tested were independently prognostic from one another. PlGF and VEGF-A were prognostic for OS (PlGF, HR, 1.31; 95% CI, 1.05–1.63; $P = 0.0166$; VEGF-A, HR, 1.10; 95% CI, 1.02–1.19; $P = 0.0143$; Supplementary Table S3), but not for PFS (PlGF, HR, 1.16; 95% CI, 0.95–1.41; $P = 0.1473$; VEGF-A, HR, 1.06; 95% CI, 0.99–1.14; $P = 0.0811$; Supplementary Table S4).

Predictive analyses

We identified PlGF as the only predictive biomarker of PFS benefit in the overall population. A high baseline level of PlGF was associated with better PFS when treated by cetuximab (BEV arm: HR, 1.51; 95% CI, 1.10–2.06; Cetux arm: HR, 0.94; 95% CI, 0.71–1.25; P_{intx}, 0.0298; **Table 2**). No other biomarkers were observed to be predictive of OS or PFS in the chemotherapy-pooled populations (Supplementary Tables S5 and S6). A Kaplan–Meier curve illustrating the predictive effect of PlGF is shown in **Fig. 3**. Patients with lower than median PlGF exhibited longer PFS and benefited more from bevacizumab than cetuximab.

To control for the possibility of the interaction effects across chemotherapies used in CALGB 80405, the biomarkers were reanalyzed for predictive associations within chemotherapy subpopulations. In patients receiving FOLFOX, low VEGF-D was associated with better PFS and benefit from bevacizumab (BEV arm: HR, 1.70; 95% CI, 1.19–2.42; Cetux arm: HR, 0.92; 95% CI, 0.68–1.24; $P_{intx} = 0.0097$, **Table 2**). Although a borderline trend was noted for PlGF, the PFS benefit did not reach statistical significance ($P_{intx} = 0.0656$, **Table 2**). Kaplan–Meier curves showed the predictive effects of VEGF-D in chemotherapy combined, as well as in FOLFOX and FOLFIRI subpopulation, respectively (**Fig. 4**). No other biomarkers were observed to be predictive for OS or PFS when patients were stratified by chemotherapy (Supplementary Tables S7 and S8).

Table 2. Predictive effects of PlGF and VEGF-D.

	OS				PFS		
	Chemo pooled				Chemo pooled		
Biomarker	Bev	Cetux	P_{intx}		Bev	Cetux	P_{intx}
PlGF	1.65 (1.16–2.36)	1.12 (0.83–1.51)	0.1157	PlGF	1.51 (1.10–2.06)	0.94 (0.71–1.25)	0.0298
VEGF-D	1.31 (0.98–1.74)	1.12 (0.84–1.50)	0.4346	VEGF-D	1.34 (1.03–1.73)	0.97 (0.75–1.27)	0.1069
	FOLFIRI				FOLFIRI		
PlGF	1.47 (0.74–2.94)	0.84 (0.45–1.57)	0.2079	PlGF	1.34 (0.71–2.51)	0.80 (0.46–1.41)	0.2487
VEGF-D	0.84 (0.49–1.45)	1.10 (0.54–2.26)	0.5643	VEGF-D	0.95 (0.61–1.48)	1.37 (0.73–2.56)	0.4336
	FOLFOX				FOLFOX		
PlGF	1.71 (1.13–2.59)	1.23 (0.88–1.71)	0.2540	PlGF	1.56 (1.08–2.24)	0.99 (0.72–1.37)	0.0656
VEGF-D	1.65 (1.17–2.33)	1.12 (0.81–1.54)	0.1151	VEGF-D	1.70 (1.19–2.42)	0.92 (0.68–1.24)	0.0097

Note: Median OS or PFS times are presented in months, with 95% CIs in parenthesis.
Abbreviations: Bev, bevacizumab; Cetux, cetuximab; P_{intx}, interaction P value.

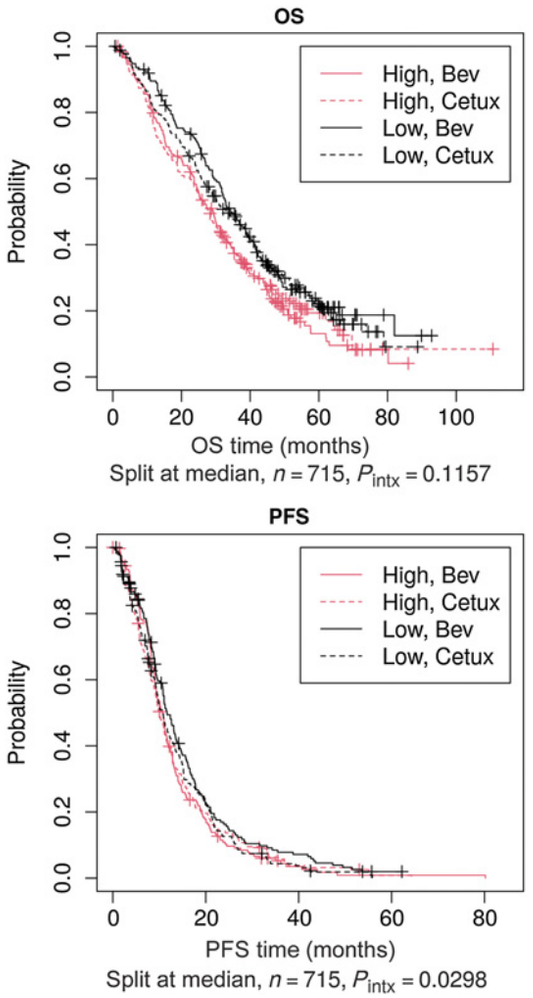

Figure 3.

Kaplan–Meier curves of PlGF and treatment for OS (upper) and PFS (bottom). For illustrative purposes, PlGF levels were dichotomized as "high" or "low" relative to the median. *P* values from the test for interaction of the marker as a continuous measure are reported.

To optimize the cut-point of VEGF-D level, Forest plots were generated to illustrate the effects of VEGF-D by quartile on OS and PFS in patients receiving FOLFOX (**Fig. 5**). Patients with VEGF-D levels in the lowest quartile had better OS (HR, 0.62; 95% CI, 0.41–0.92) and PFS (HR, 0.59; 95% CI, 0.41–0.85).

Discussion

CALGB 80405 is the largest phase III trial to directly compare bevacizumab and cetuximab, two of the most commonly used targeted agents, in addition to FOLFIRI or FOLFOX as first-line treatment in advanced or mCRC. Blood samples collected during CALGB 80405 provide a unique and valuable source of materials for biomarker studies that may help guide the use of these therapeutics. Our retrospective biomarker analysis identified PlGF and VEGF-D, two chemo-dependent predictive biomarkers, as well as numerous prognostic biomarkers.

We identified 10 biomarkers with highly consistent prognostic impact across both OS and PFS. These biomarkers play important roles in angiogenesis (Ang-2, HGF, TSP-2, VEGF-R3), inflammatory and immune modulating (CD73, IL6, ICAM-1, VCAM-1), and extracellular matrix remodeling (OPN, TIMP-1). Intrinsic high expression of all these biomarkers was associated with worse outcomes, reflecting the coordinated interplay of angiogenesis, inflammation, and immune modulation. Interestingly, PlGF and VEGF-A were found to be prognostic for OS, but not for PFS. This is likely due to the manifest of stronger prognostic effect at later stage of the disease or over longer time, as the median OS was approximately 20 months longer than median PFS (25).

With respect to EGFR inhibition, we previously identified two predictive biomarkers for cetuximab benefit (HER-3 and CD73) across all patients (*KRAS* mutant and wild-type) in CALGB 80203 (28), a randomized study evaluating the efficacy of cetuximab in combination with FOLFOX or FOLFIRI in mCRC. However, neither biomarker predicted benefit from cetuximab here in CALGB 80405 (Supplementary Tables S5–S8). HER-3 belongs to the same family of receptor tyrosine kinases as EGFR, with the potential to serve as a resistance mechanism to cetuximab (32). CD73, an immune modulatory AMP phosphatase, was associated with cetuximab benefit in previous studies (28, 33). The lack of predictive value in this study may be due to the potential association between CD73 and *RAS* mutational status (34). In this correlative study, all patients included were *KRAS* wild type at codons 12 and 13. Exclusion of patients with *RAS* mutations may confound the analysis. Although *RAS* wild-type has been widely applied as a prerequisite for patients to receive anti-EGFR inhibitors, not all patients with wild-type *RAS* respond to EGFR inhibitors. Our Angiome analysis could provide novel insight regarding the patient population most likely to derive benefit from the EGFR-targeting drugs.

With respect to VEGF inhibition, VEGF-A has long been considered a candidate for predictive biomarker for anti-angiogenic therapies, and high levels of VEGF-A may indicate increased biological dependency on this angiogenic factor. Yet, a meta-analysis of more than 1,800 patients participating in phase III trials in colorectal cancer, non–small cell lung cancer, and renal cell carcinoma confirmed that pretreatment VEGF-A levels have only prognostic, but not predictive value (35). In the MERiDiAN trial, preselecting metastatic breast cancer patients based on high plasma levels of short isoforms of VEGF-A for bevacizumab treatment revealed no significant association of PFS by VEGF-A interaction, failing to support using baseline VEGF-A to identify patients benefitting most from bevacizumab (36). Similarly, PlGF has prognostic value, but has not been shown to be predictive for anti-angiogenic therapy (37). Our data provide novel insight into the potential predictive role of these VEGF family members.

In this study, we found PlGF was predictive of lack of PFS benefit from bevacizumab independent of the type of chemotherapy used (**Table 2**; **Fig. 3**). PlGF belongs to the VEGF family; it binds and activates signaling through VEGF-R1, but does not directly signal through VEGF-R2 (38). PlGF modulates VEGF-A signaling both at the ligand level (by forming heterodimers with VEGF-A) and the receptor level (by engaging VEGF-R1; ref. 39). PlGF has been noted as a pharmacodynamic biomarker with circulating levels consistently increasing in response to anti-VEGF agents (22, 40, 41), but this is the first report of a potential predictive association between baseline PlGF levels and outcome in colorectal cancer. Patients with lower than median levels of PlGF derived more PFS benefit from bevacizumab

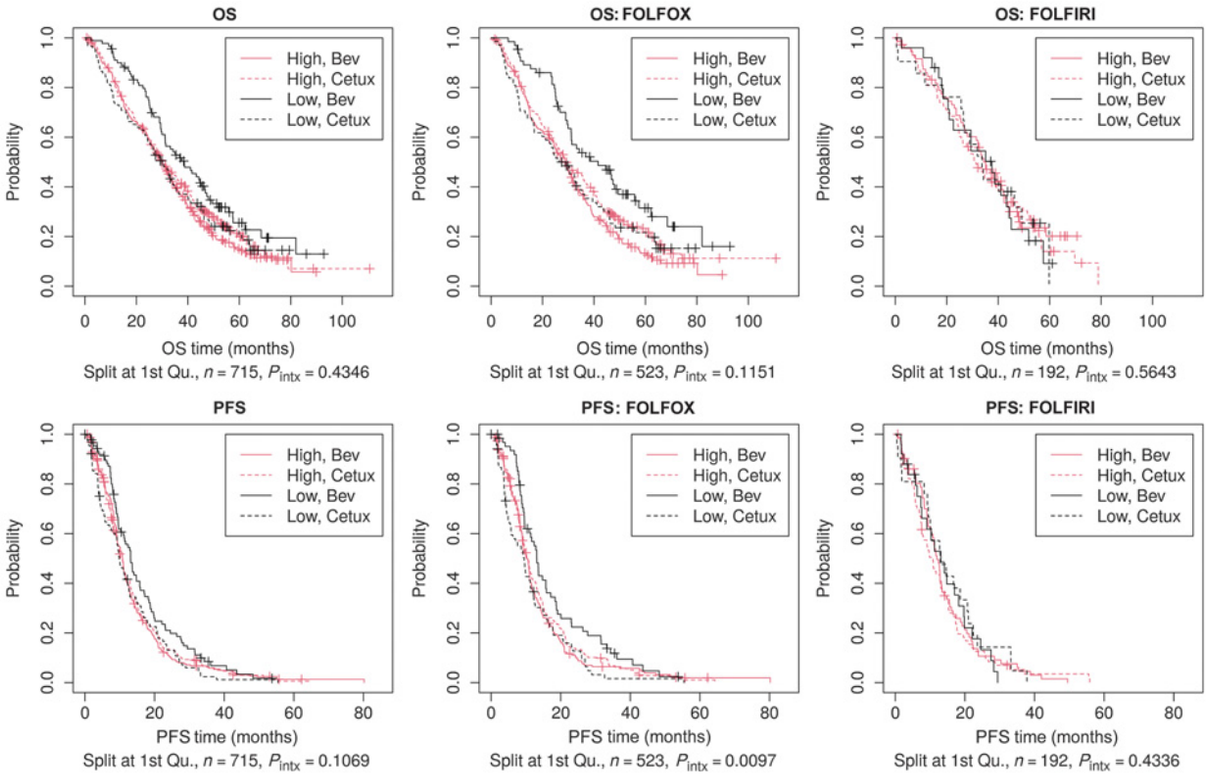

Figure 4.

Kaplan–Meier curves showing the effects of VEGF-D levels and chemotherapy on OS (upper) and PFS (bottom). For illustrative purposes, VEGF-D levels were dichotomized as "high" or "low" relative to the first quartile. *P* values from the test for interaction of the marker as a continuous measure are reported. Patients with VEGF-D levels in the lowest quartile receiving FOLFOX as the chemotherapy derived more benefit from bevacizumab (the interaction *P* value for the marker as a continuous measure was 0.0097), as shown in the middle bottom figure.

($P_{intx} = 0.0298$, **Fig. 3**), independent of chemotherapy received. It is important to note that the comparative arm used in this study is an active therapy (cetuximab) and not a true placebo. This may explain why we observed PlGF as a predictive biomarker only in this specific study population.

VEGF-D was identified as the strongest predictive biomarker for lack of PFS benefit from bevacizumab; however, this was only observed in the FOLFOX treated patients ($P_{intx} = 0.0097$, **Table 2**). VEGF-D is another VEGF family member; it binds VEGF-R2 promoting angiogenesis, as well as VEGF-R3 where it promotes lymphangiogenesis (42). The predictive effect was largest in patients with VEGF-D levels in the lowest quartile, where patients derived the most benefit from bevacizumab (**Fig. 5**). Importantly, far more patients in CALGB 80405 study received FOLFOX ($n = 523$) than FOLFIRI ($n = 192$). It is possible that additional clinical factors related to the use of FOLFOX over FOLFIRI may confound the VEGF-D effect observed here. The exact mechanism for this chemotherapy effect remains unclear. The VEGF-D effect is consistent with results seen previously in patients with pancreatic cancer treated with bevacizumab and gemcitabine in CALGB 80303 (20), and in patients with colorectal cancer treated in the Australian Gastro-Intestinal Trials Group testing Mitomycin, Avastin, and Xeloda (AGITG MAX trial; ref. 23). In all cases, low VEGF-D predicts benefit from bevacizumab, while high VEGF-D predicts for lack of benefit from bevacizumab. Excessive VEGF-D can provide compensatory signaling in the VEGF axis through VEGF-R2, serving as a resistance mechanism upon VEGF-A blockage by bevacizumab. Therefore, patients are less likely to benefit from bevacizumab given the presence of a readily available compensatory mechanism. The predictive roles of PlGF and VEGF-D noted here in CALGB 80405 may suggest that alternate VEGF family members may mediate intrinsic resistance mechanisms to bevacizumab, which selectively binds VEGF-A.

Although no other biomarkers reach statistical significance, a few interesting trends were noted. First, VEGF-C, an additional VEGF family member with potent angiogenic and lymphangiogenic properties, appears to predict for OS benefit from cetuximab in FOLFIRI subgroup ($P_{intx} = 0.0791$, Supplementary Table S7). In addition, in the subgroup of patients receiving FOLFIRI, we identified three TGFβ-related biomarkers to be potentially predictive of PFS benefit, including TGFβ1 ($P_{intx} = 0.0524$), PDGF-AA ($P_{intx} = 0.0511$), and SDF-1 ($P_{intx} = 0.0688$; Supplementary Table S8). Higher levels of TGFβ1 and PDGF-AA potentially predict for benefit from cetuximab. These biomarkers may reflect overlapping biologies contributing to cetuximab sensitivity. Interplay between EGFR and TGFβ signaling pathways have been reviewed extensively (43).

Despite extensive effort, no predictive biomarker has been identified that would enable a more personalized use of bevacizumab. Angiogenesis is a complex process involving diverse cell types, cytokines, and growth factors signaling through multiple pathways. The biological redundancies eventually lead to anti-angiogenic drug resistance. As the biology of angiogenesis and its role in tumor development varies across tumor types, suitable biomarkers may be tumor type specific. Although

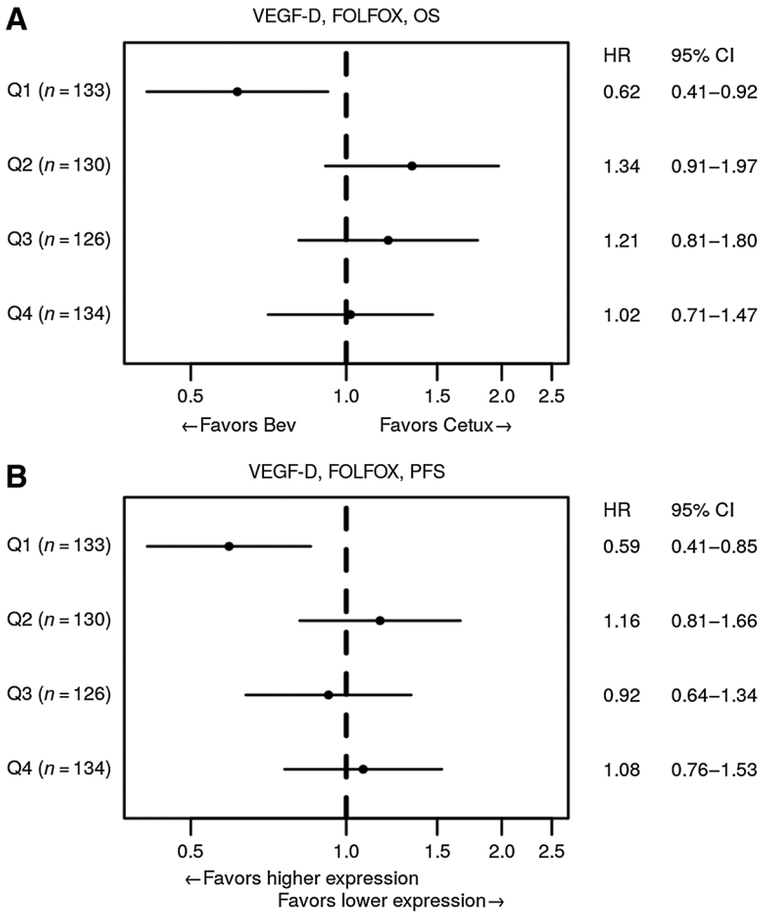

Figure 5.
Predictive effect of VEGF-D. Forest plots of VEGF-D quantiles in the FOLFOX-treated subpopulation for OS (**A**) and PFS (**B**). Patients with VEGF-D levels in the lowest quantile derived benefit from bevacizumab.

angiogenic factors (PlGF and VEGF-D) were noted to be associated with PFS benefit in patients with mCRC in CALGB80405, other studies noted the predictive potential of the inflammatory cytokine IL6 in the context of anti-angiogenic therapy in renal cancer and ovarian cancer (21, 44).

This analysis has several limitations. The patients receiving FOLFIRI were much fewer than those receiving FOLFOX, therefore underpowered to explore key subgroups. The unbalanced use of FOLFIRI and FOLFOX may introduce uncontrolled bias into these analyses. Although all patients are considered *KRAS* WT, *KRAS* testing was only performed at codons 12 and 13. It is possible that some patients with *KRAS* mutant were included in our analysis. Genetic information such as extended *RAS* and *RAF* mutations (45), microsatellites instability and tumor mutational burdens (46), consensus molecular subtypes (CMS), representing intrinsic heterogeneity of colorectal cancer at the genetic expression level (47) will be further investigated in subsequent analysis. In addition, this retrospective biomarker analysis was intended to be exploratory and hypothesis-generating in nature, hence the *P* values presented here were not adjusted for multiple testing. The current findings are promising and biologically plausible but should be considered preliminary and not ready to guide patient care at this time. To advance any of these putative biomarkers along the validation path, a predefined cut-point would need to be established and tested. Ideally, any biomarker cut-point would be tested prospectively as a stratification factor or for entry onto the trial. The establishment of a cut-point is a crucial step for clinical decision making when considering the prospective use of any prognostic or predictive biomarker. As such, validation of VEGF-D as a biomarker with a specific cut-point awaits further investigation.

Discovery and validation of predictive biomarkers is challenging and requires a detailed understanding of the target expression and biology in patients, technical optimization of biomarker assays, and clinical validation of any findings. This analysis in CALGB 80405 is the culmination for nearly two decades of work by this group. The findings of predictive biomarkers may be applied to other FDA-approved drugs for mCRC. Notably, ziv-aflibercept, a recombinant receptor containing the ligand-binding domains of VEGF-R1 and VEGF-R2, binds both VEGFs and PlGF (15, 48); while ramucirumab blocks the binding of both VEGF-A, -C, and -D to VEGF-R2 (49, 50). It is not known whether these agents may be more active in patients with higher levels of plasma PlGF and VEGF-D. Nevertheless, our data support the rationale for biomarker-driven clinical studies to address this possibility.

In conclusion, our biomarker analysis of CALGB 80405 identified two candidate predictive biomarkers for lack of benefit from bevacizumab: PlGF and VEGF-D. Future studies to validate and refine these findings are warranted.

Authors' Disclosures

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Authors' Contributions

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Note

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