

Phase II Trial of Infusional Fluorouracil, Irinotecan, and Bevacizumab for Metastatic Colorectal Cancer: Efficacy and Circulating Angiogenic Biomarkers Associated With Therapeutic Resistance

Scott Kopetz, Paulo M. Hoff, Jeffrey S. Morris, Robert A. Wolff, Cathy Eng, Katrina Y. Glover, Rosie Adinin, Michael J. Overman, Vincete Valero, Sijin Wen, Christopher Lieu, Shaoyu Yan, Hai T. Tran, Lee M. Ellis, James L. Abbruzzese, and John V. Heymach

From The University of Texas M.D. Anderson Cancer Center; Lyndon B. Johnson Hospital, Harris County Hospital District, Houston, TX; and Instituto do Cancer do Estado de Sao Paulo, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, Brazil.

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Corresponding author: Scott Kopetz, MD, 1515 Holcombe Blvd, Unit 426, Houston, TX 7703; e-mail: skopetz@mdanderson.org.

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ABSTRACT

Purpose

We investigated the efficacy of fluorouracil (FU), leucovorin, irinotecan, and bevacizumab (FOLFIRI + B) in a phase II trial in patients previously untreated for metastatic colorectal cancer (mCRC), and changes during treatment in plasma cytokines and angiogenic factors (CAFs) as potential markers of treatment response and therapeutic resistance.

Patients and Methods

We conducted a phase II, two-institution trial of FOLFIRI + B. Each 14-day cycle consisted of bevacizumab (5 mg/kg), irinotecan (180 mg/m²), bolus FU (400 mg/m²), and leucovorin (400 mg/m²) followed by a 46-hour infusion of FU (2,400 mg/m²). Levels of 37 CAFs were assessed using multiplex-bead assays and enzyme-linked immunosorbent assay at baseline, during treatment, and at the time of progressive disease (PD).

Results

Forty-three patients were enrolled. Median progression-free survival (PFS), the primary end point of the study, was 12.8 months. Median overall survival was 31.3 months, with a response rate of 65%. Elevated interleukin-8 at baseline was associated with a shorter PFS (11 v 15.1 months, $P = .03$). Before the radiographic development of PD, several CAFs associated with angiogenesis and myeloid recruitment increased compared to baseline, including basic fibroblast growth factor ($P = .046$), hepatocyte growth factor ($P = .046$), placental growth factor ($P < .001$), stromal-derived factor-1 ($P = .04$), and macrophage chemoattractant protein-3 ($P < .001$).

Conclusion

Efficacy and tolerability of FOLFIRI + B appeared favorable to historical controls in this single arm study. Before radiographic progression, there was a shift in balance of CAFs, with a rise in alternate pro-angiogenic cytokines and myeloid recruitment factors in subsets of patients that may represent mechanisms of resistance.

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INTRODUCTION

Therapies incorporating the monoclonal antibody bevacizumab, an inhibitor of vascular endothelial growth factor (VEGF), have demonstrated efficacy in metastatic colorectal cancer.¹⁻³ The majority of previously untreated metastatic colorectal cancer patients are treated with bevacizumab (B) in combination with oxaliplatin and fluorouracil (FOLFOX).⁴ Although the literature suggests an equivalent efficacy for FOLFIRI and FOLFOX without a monoclonal antibody, there are

few reports on the FOLFIRI + B regimen in previously untreated patients.^{5,6}

Despite the benefit provided by bevacizumab-based regimens for patients with metastatic colorectal cancer, clinical resistance usually develops. Extensive preclinical work has suggested that alternate proangiogenic factors may modulate sensitivity to anti-VEGF therapy and allow regrowth of tumor-associated vasculature.⁷ Additional studies have implicated infiltrating monocytic cells in the angiogenic switch, recruited by cytokines derived from tumor or tumor-associated

stroma.⁸ However, clinical studies incorporating analysis of these potential cytokines are limited by the number and time points of collected samples.

This phase II study we report here was designed to determine the efficacy of the FOLFIRI + B regimen and to explore both predictors of sensitivity and potential mechanisms of resistance to FOLFIRI + B. We report the clinical efficacy of this regimen and identified elevation of several proangiogenic cytokines before, and at the time of, progression on this regimen.

PATIENTS AND METHODS

Patients and Eligibility Criteria

Patients were enrolled from M. D. Anderson Cancer Center and Lyndon B. Johnson General Hospital, a county hospital affiliated with The University of Texas, Houston, TX. Enrollment began in January 2005 and completed in January 2007.

Eligible patients were ≥ 18 years old, and were required to have histologically confirmed colorectal cancer with measurable metastatic disease per RECIST (Response Evaluation Criteria in Solid Tumors), with no prior chemotherapy for metastatic disease and with at least 6 months elapsed from completion of any adjuvant therapy. All patients had adequate hepatic, renal, and marrow functions, and Eastern Cooperative Oncology Group performance status of ≤ 2 . Written informed consent was obtained from each patient after approval of the clinical study from both institutional review boards.

Treatment

The FOLFIRI + B regimen consisted of bevacizumab (5 mg/kg), irinotecan (180 mg/m²), bolus fluorouracil (400 mg/m²), and leucovorin (400 mg/m²), followed by a 46-hour infusion of fluorouracil (2,400 mg/m²). Patients were treated with bevacizumab alone on day 1, starting FOLFIRI + B on day 15. This single dose of bevacizumab was administered to allow correlative studies to be completed before and after bevacizumab alone.

Dose reductions were required for all grade 3 or 4 toxicities attributed to study medications. Bevacizumab was not dose reduced. Treatment was continued until disease progression, unacceptable toxicities, or withdrawal of consent. Adverse event grading was performed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

Statistical Analysis

The primary objective of the trial was PFS, with a null hypothesis of an 8 months PFS for FOLFIRI alone.⁹⁻¹¹ The target enrollment of 43 patients provided an 80% power to detect a 4-month improvement in PFS, with an α of .05.² The secondary clinical objectives included evaluation of response rate, overall survival, and toxicity. PFS was defined as time from study enrollment to progression of disease or death, without censoring for treatment discontinuation. Overall survival was defined as the time from study enrollment to death from any cause.

Plasma Sample Collection and Analysis

Venous blood was drawn and immediately processed for plasma at baseline, immediately before each cycle of chemotherapy (including a sample 2 weeks after single-agent bevacizumab in the first cycle), and, when available, at the time of progression. On retrospective review of computed tomography imaging, we identified the plasma sample associated with the best radiographic response for all patients. This sample (henceforth denoted as "before progression") represented a point before the development of progressive disease by RECIST, which occurred a median of 8 weeks later. Plasma was stored at -80°C until analysis.

Each sample was analyzed in duplicate and as previously described with no more than one prior freeze-thaw cycle.¹² Thirty-seven factors with angiogenic and/or monocyte recruitment activity and present on commercially available multiplex bead suspension bead arrays (Biosource, Camarillo, CA;

LINCO Research, St Charles, MO) and enzyme-linked immunosorbent assays (vascular endothelial growth factor [VEGF], soluble VEGFR2 [sVEGFR2], placental growth factor [PIGF]; R&D Systems, Minneapolis, MN) were analyzed per the manufacturers' directions (factors listed in Appendix Table A1, online only, and grouped by class). Samples were allocated onto the arrays using a randomized block design. The relationship between continuous variables was assessed by Spearman correlation. Survival and progression-free survival distributions were summarized by Kaplan-Meier methods and compared using log-rank tests. All *P* values are two sided.

Using Wilcoxon tests, we assessed which factors differed significantly between different time points, specifically from baseline (pretreatment) until after (A) bevacizumab, (B) after bevacizumab + FOLFIRI, (C) before progression, and (D) after progression, plus comparisons from (E) bevacizumab + FOLFIRI to best before progression, and from (F) before progression until after progression, and from (G) bevacizumab + FOLFIRI to progression. Given the large number of comparisons, we adjusted for multiple testing using the false discovery rate methods, which is a standard multiple test adjustment procedure.¹³ Specifically, we apply the *fdrtool* method to map each *P* value to a *q* value, which can be interpreted as the probability that the given factor is a false discovery.^{14,15} We flagged as significant any factor with *q* value less than 0.05, meaning that we are at least 95% certain it is a true discovery.

RESULTS

Baseline Characteristics

Forty-three patients were enrolled, as shown in Figure 1. The median age of the patients was 57 years (range, 26 to 78), and 41% were female. Fourteen percent of patients self-reported to be of Hispanic ethnicity, 10% African American, and 74% white. Eastern Cooperative Oncology Group performance status scores were 0, 1, and 2 in 42%, 56%, and 2% of the patients, respectively. Primary tumors were graded as poorly differentiated in 14% and moderately differentiated in the remainder. Paired plasma samples were obtained for 40 patients at baseline, after single-agent bevacizumab, after FOLFIRI + B, and before progression. Nineteen samples were available at the time of radiographic progression. The number of samples varied due to logistic failure to obtain all samples or patient withdrawal for reasons besides progression.

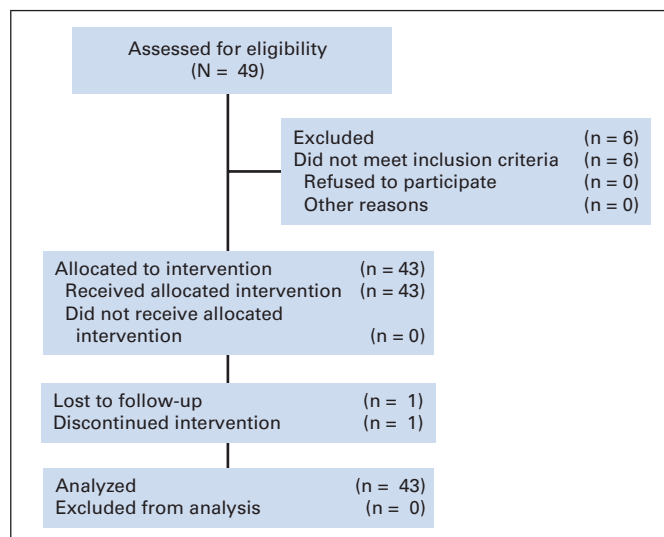


Fig 1. CONSORT diagram.

Efficacy

The median PFS, the primary study end point, was 12.8 months, as shown in Figure 2A. Median overall survival was 31.3 months (Fig 2B). On-treatment PFS, in which patients whose initial treatment ceased without progression were censored at the date of last computed tomography scan, was 10.2 months.

Twenty-eight patients had confirmed partial responses, for an overall response rate of 65% (95% CI, 49% to 79%). One additional

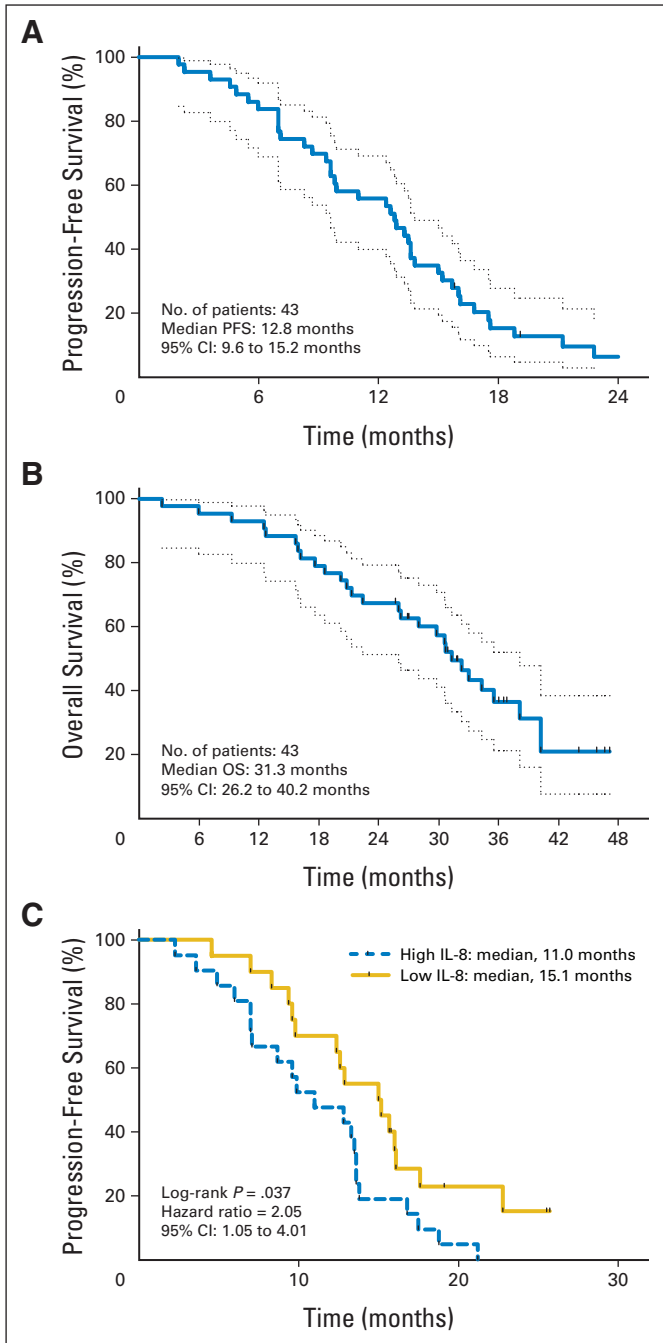


Fig 2. Kaplan-Meier plots. (A) Progression-free survival and (B) overall survival for patients treated with FOLFIRI + B. The 1-year and 2-year survivals were 93% (95% CI, 98% to 80%) and 65% (95% CI, 78% to 48%), respectively. (C) Progression-free survival for patients with interleukin-8 levels above and below the median value of 3.7 pg/mL.

patient had an unconfirmed partial response. Responses were observed at a median of 4.1 months, with 23%, 28%, 9%, and 5% of patients obtaining a response on the first, second, third, and fourth or later restaging studies, respectively (Appendix Fig A1, online only). Four patients were subsequently able to undergo resection of metastatic disease.

Toxicities

Fifty-six percent of patients required dose reductions for toxicity during the course of treatment. The rate of grade 3 or 4 toxicities was low, as presented in Table 1. Eight patients stopped treatment for toxicities. Neutropenia was the most common hematologic adverse event, with rare febrile neutropenia. Six patients had grade 2 diarrhea. Adverse events related to bevacizumab included bleeding, proteinuria, and hypertension. In addition, there was one possible localized bowel perforation managed conservatively. All patients had their blood pressure controlled, requiring a median of two blood pressure medications. Nasal epistaxis was seen in 45% patients, associated with mild nasal mucosal ulceration in most cases. Five patients stopped protocol therapy for surgical procedures. No wound healing complications were noted.

Cytokines and Angiogenic Factors at Baseline and After Initial Treatment

In the 40 evaluable patients, neither the baseline levels of VEGF nor those of VEGFR2 were associated with differences in PFS or overall survival. However, when dichotomized at the median value (3.7 pg/mL), high interleukin (IL)-8 levels were associated with a shorter PFS (11 v 15.1 months; $P = .03$), and were correlated with increased tumor volume (Spearman $r = .62$; $P < .001$; Fig 2C). A similar correlation was found between tumor volume and baseline VEGF, hepatocyte growth factor (HGF), and E-selectin, although none of these were correlated with subsequent outcomes after treatment (Appendix Fig A2, online only).

The modulation of each marker after treatment with single-agent bevacizumab and after one cycle FOLFIRI + B was evaluated. When

Table 1. Adverse Events

Adverse Event	Grade 3 or 4		All Grades	
	No. of Patients	%	No. of Patients	%
Neutropenia	17 (3 grade 4)	40	27	64
Hypertension	8	19	15	36
Venous thromboembolism	8 (5 grade 4)	19	12	29
Fatigue	6	14	38	90
Diarrhea	1	2	30	71
Vomiting	1	2	25	60
Hemoglobin	1	2	22	52
Neutropenic fever	1	2	1	2
Alopecia	—	—	30*	71*
Nose bleed/nasal septal ulceration	0	0	19	45
Mucositis	0	0	19	45
Proteinuria	0	0	13	31
Constipation	0	0	9	21
Anorexia	0	0	5	12

NOTE. No deaths occurred on treatment or within 28 days of study discontinuation.
 *Eight (19%) patients had grade 2 alopecia.

compared with baseline levels, treatment with single-agent bevacizumab revealed an increase in PIGF ($P = .01$), eotaxin ($P = .01$), and sVEGFR2 ($P = .03$), and a decrease in E-selectin ($P < .001$). After one cycle of FOLFIRI + B, these same changes in PIGF, eotaxin, sVEGFR2, and E-selectin continued; in addition, matrix metalloproteinase-9 and IL-8 levels were reduced ($P < .001$ and $P = .01$, respectively; Appendix Table A2).

Cytokine and Angiogenic Factor Modulation Before and at Progression

In order to understand the dynamic changes in circulating factors during the emergence of therapeutic resistance, we evaluated profiles of cytokines and angiogenic factors before progression, when we hypothesized that angiogenic activity may have been restarting, and at the time of radiographic progression. Several proangiogenic cytokines were elevated before progression, notably basic fibroblast growth factor (bFGF), PIGF, and HGF (Fig 3). At the time of radiographic progression, bFGF, and MMP-9 were increased compared with the levels after a single dose of FOLFIRI + B ($P < .001$ for both), although HGF was no longer elevated. At the time of radiographic progression,

sVEGFR2 decreased to below baseline levels ($P = .005$). Compared with levels after FOLFIRI + B, PDGF levels were increased before progression ($P = 0.001$). In a sensitivity analysis, these same trends persisted when analysis was limited to patients with samples available for all five time points (data not shown).

Changes in some of the cytokines were correlated with each other (eg, bFGF, IL-8, E-selectin, and PDGF), although there was a wide spectrum of modulation between patients (Appendix Fig A3, online only). Despite this large interpatient variability, a subset of patients had no discernable increase in alternate/redundant proangiogenic factors before progression or at the time of progression.

Because myeloid cell recruitment has been hypothesized to play a role in resistance to antiangiogenic therapy, we analyzed cytokines with known roles in myeloid cell differentiation and recruitment. Before progression, there was an increase in SDF-1 ($P = .038$) and MCP-3 ($P < .001$), two factors known to be potent chemoattractants for myeloid cells and hematopoietic progenitor cells. Intriguingly, several factors associated with eosinophil recruitment and differentiation were also elevated before progression and at the time of progression. Eotaxin, the level of which was increased by single-agent

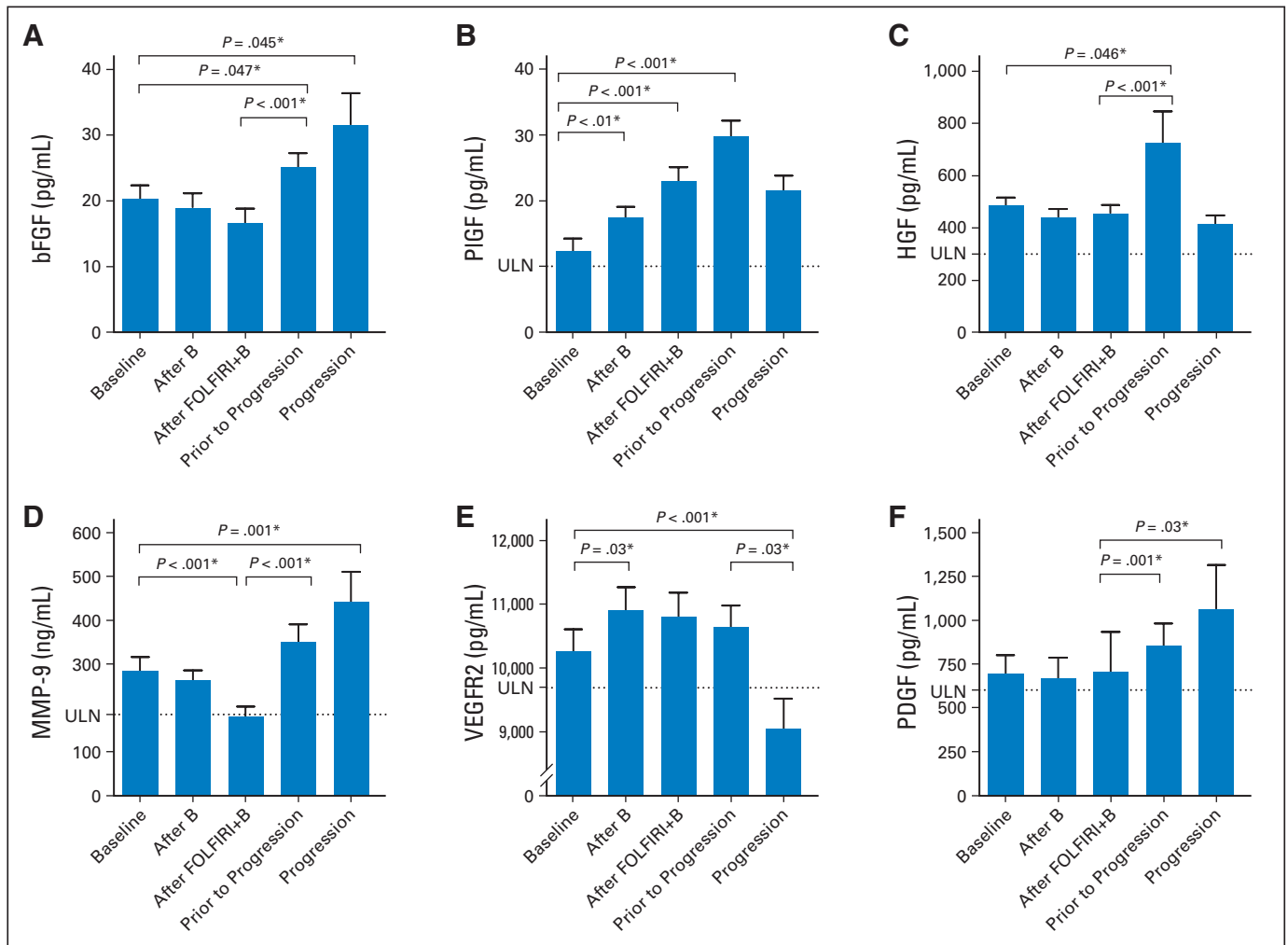


Fig 3. Cytokine values at baseline, after a single dose of bevacizumab, after a single dose of fluorouracil, leucovorin, irinotecan, and bevacizumab (FOLFIRI + B), before progression, and at the time of progression. (*) Significantly different after multiple comparison correction, with significance defined by local false discovery rate q less than 0.05. ULN, upper limit of normal.

bevacizumab, was further increased before progression and at the time of progression ($P < .001$, for both). Similarly, IL-16, IL-18, and macrophage inhibitory factor were also higher at the time of progression than at baseline (Appendix Fig A4, online only).

DISCUSSION

The progression-free survival of 12.8 months and response rate of 65% for FOLFIRI + B compares favorably with historical controls, with low rates of toxicity. A previously published study has demonstrated the efficacy of FOLFIRI when combined with bevacizumab in 57 patients, although there are no published results of larger prospective studies of this regimen despite its widespread use.^{1,16} The overall survival of 31.3 months compares favorably with 20.3 months reported with bolus fluorouracil, leucovorin, irinotecan, and bevacizumab and 16 to 23 months reported with FOLFIRI alone.^{1,2,17} Unlike the older irinotecan and bolus fluorouracil regimens, which resulted in severe diarrhea and neutropenia, FOLFIRI + B is well tolerated.¹⁸

The cytokine analyses demonstrated that alternate proangiogenic cytokines are modulated by bevacizumab and FOLFIRI + B, and that they are increased before disease progression in a subset of patients receiving this bevacizumab-containing regimen. Single-agent bevacizumab and FOLFIRI + B resulted in lower average levels of bFGF, MMP-9, IL-8, and E-selectin. Before progression, mean bFGF, PlGF, MMP-9, PDGF, and HGF increased, among others. In addition, a subset of factors associated with monocyte recruitment was elevated at disease progression.

The mean bFGF level decreased after one cycle of FOLFIRI + B but was elevated before and at the time of progression. In preclinical studies, bFGF has been shown to be a compensatory angiogenic factor after VEGF inhibition, resulting in renewed angiogenesis independent of VEGF.^{19,20} Previously, bFGF has been shown to increase at the time of progression in patients with glioblastoma treated with the VEGF receptor tyrosine kinase inhibitor cediranib.²¹ However, a study of bevacizumab with or without interferon- α 2b in melanoma patients did not demonstrate any changes in bFGF levels during treatment.²² Similarly, modulation of bFGF was not seen after a limited course of single-agent bevacizumab in rectal cancer.²³

PlGF, a ligand for VEGFR1 that is involved with angiogenesis but not bound by bevacizumab, was increased in a subset of patients after administration of bevacizumab and FOLFIRI + B. A similar increase has been shown within weeks after initiation of a VEGF receptor tyrosine kinase inhibitor, and as soon as 2 days after administration of bevacizumab.^{21,23-26} In our study, average PlGF was highest at the point before progression and subsequently decreased with the development of radiographically evident progressive disease. This is concordant with a prior report that PlGF is decreased at the time of progression, as this study did not incorporate time points before progression.²¹ It remains unclear whether PlGF is sufficient to restore angiogenesis in the presence of VEGF inhibition, although this is suggested in preclinical models.²⁷

A similar pattern of peak average levels before the development of radiographically evident progressive disease was seen with HGF and, to a lesser extent with stem cell factor (SCF)/c-kit ligand, with average levels returning to normal at the time of radiographic progressive disease. HGF, via activation of the Met receptor, is a potent angiogenic factor that stimulates VEGF and IL-8 expression while inhibiting

negative regulators of angiogenesis.²⁸⁻³¹ The finding of high levels of PlGF, HGF, and SCF levels in a subset of patients before progression suggests that these factors, known to be regulated by HIF-1 α , may reflect a hypoxia response. As establishment of functional vasculature is a prerequisite for disease growth and predates disease progression, the hypoxic response as measured by cytokines in circulation may be resolving at the time of radiographically evident progressive disease.

Soluble VEGFR2 increased after a single dose of bevacizumab and remained elevated with continued treatment with FOLFIRI + B until decreasing below baseline on the development of progressive disease. This is in contrast to the consistent data demonstrating an initial decrease in sVEGFR2 after treatment with VEGFR TKIs with or without cytotoxic chemotherapy, with rising sVEGFR2 at the time of progression, and likely represents a distinction between the modulation profiles of bevacizumab and VEGF TKIs.^{26,32,33} In support of this, treatment with a single dose of bevacizumab resulted in an initial increase in sVEGFR2 in a neoadjuvant rectal cancer study.²³ In our study, changes in VEGFR2 were not correlated with outcomes with this regimen. MMP-9 has previously been shown to decrease after platinum-based chemotherapy with and without vandetanib in non-small-cell lung cancer patients, and rising levels on treatment were associated with tumor progression, although the latter was not seen in this study.^{12,34}

Myeloid cell recruitment to the tumor bed via several cytokines including PlGF and SDF-1 has been described previously and increased levels of these factors were seen in this study before progression.³⁵⁻³⁸ Infiltrating myeloid-lineage cells release additional proangiogenic factors and may modulate response to antiangiogenic therapies.³⁹ Interestingly, eotaxin and IL-16, both potent chemoattractants for eosinophils, were elevated in many patients at the time of progression.⁴⁰ Eosinophil granules contain multiple proangiogenic factors including VEGF and bFGF, but have not been previously associated with resistance to VEGF inhibition in the clinic.³⁹ IL-18, the expression of which is tightly correlated to IL-16 and also rises at progression, is a pro-inflammatory cytokine expressed by macrophages and monocytes and has been shown to stimulate migration of endothelial cells and induce angiogenesis *in vivo*.^{41,42}

It should be noted that this study is exploratory in nature and has several limitations. Multiple cytokines were evaluated in a modest sample size, leading to the possibility of false positive results, although multiple-comparison testing methods were utilized. Circulating cytokine levels may not reflect the local concentrations of these factors in the tumor microenvironment, and may also reflect a response of non-neoplastic, host tissue to the treatment. Additional studies will clearly be needed to determine whether specific factors associated with resistance in this study are consistent and causative of resistance to bevacizumab-containing regimens. The single-arm study design, likewise, does not allow us to distinguish between cytokine changes induced by bevacizumab and those induced by the cytotoxic chemotherapy component of the regimen. Nevertheless, the associations between specific circulating pro-angiogenic and myeloid recruitment factors and the emergence of therapeutic resistance suggest several potential mechanisms that merit further investigation.

Importantly, these results suggest that plasma cytokine levels at the time of progression are different from earlier changes before progression, when compensatory angiogenic factors may be stimulating new vessel growth in preparation for clinically evident progression. Given the lack of single-agent activity of bevacizumab, resistance that

develops to the cytotoxic components may manifest in progressing disease without an “angiogenic switch.” Indeed, approximately a third of patients did not have an identifiable increase in alternate angiogenic factors before progression. It remains an intriguing hypothesis that similar analyses might identify a subset of patients progressing on a bevacizumab regimen that may continue to benefit from further bevacizumab therapy.^{43,44} Conversely, one could speculate that those patients with significant elevations in PlGF, bFGF, or HGF at progression might not benefit from continuation of bevacizumab in second-line therapy, but may benefit from further antiangiogenic therapy using VEGFR TKIs, bFGF inhibitors, or MET inhibitors, respectively. Further studies are planned to explore this speculation, validate these findings, and to better quantify the apparent interpatient heterogeneity of cytokine changes.

In conclusion, this study demonstrates the long progression-free and overall survival of patients who received the FOLFIRI + B regimen. Elevations of pro-angiogenic cytokines, notably bFGF, PlGF, and HGF, were observed in subsets of patients before radiographic evidence of disease progression. Prospective measurements of these or other pro-angiogenic cytokines may provide a mechanism to individualize continued antiangiogenic therapy after progression on a bevacizumab-containing regimen.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a “U” are those for which no compensation was received; those relationships marked with a “C” were compensated. For a detailed description of the disclosure categories, or for more information about

ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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AUTHOR CONTRIBUTIONS

Conception and design: Scott Kopetz, Paulo M. Hoff, James L. Abbruzzese, John V. Heymach

Financial support: James L. Abbruzzese, John V. Heymach

Administrative support: Scott Kopetz, Christopher Lieu, James L. Abbruzzese

Provision of study materials or patients: Scott Kopetz, Robert A. Wolff, Cathy Eng, Katrina Y. Glover, Vicente Valero

Collection and assembly of data: Scott Kopetz, Rosie Adinin, Vicente Valero, Shaoyu Yan, Hai T. Tran, John V. Heymach

Data analysis and interpretation: Scott Kopetz, Jeffrey S. Morris, Katrina Y. Glover, Michael J. Overman, Sijin Wen, Christopher Lieu, Shaoyu Yan, Hai T. Tran, Lee M. Ellis, John V. Heymach

Manuscript writing: Scott Kopetz, Paulo M. Hoff, Michael J. Overman, Lee M. Ellis, John V. Heymach

Final approval of manuscript: Scott Kopetz, Paulo M. Hoff, Jeffrey S. Morris, Robert A. Wolff, Cathy Eng, Katrina Y. Glover, Rosie Adinin, Michael J. Overman, Vicente Valero, Shaoyu Yan, Hai T. Tran, Lee M. Ellis, James L. Abbruzzese, John V. Heymach

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