

Association between VEGF Splice Isoforms and Progression-Free Survival in Metastatic Colorectal Cancer Patients Treated with Bevacizumab

David O. Bates¹, Paul J. Catalano³, Kirsty E. Symonds¹, Alex H.R. Varey¹, Pramila Ramani², Peter J. O'Dwyer⁴, Bruce J. Giannone⁴, Neal J. Meropol⁵, Al Bowen Benson⁶, and Steven J. Harper¹

Abstract

Purpose: Bevacizumab improves survival for patients with metastatic colorectal cancer with chemotherapy, but no proven predictive markers exist. The VEGF-A splice form, VEGF_{165b}, anti-angiogenic in animal models, binds bevacizumab. We tested the hypothesis that prolonged progression-free survival (PFS) would occur only in patients with low relative VEGF_{165b} levels treated with bevacizumab.

Experimental Design: Blinded tumor samples from the phase III trial of FOLFOX4 ± bevacizumab were assessed for VEGF_{165b} and VEGF_{total} by immunohistochemistry and scored relative to normal tissue. A predictive index (PI) was derived from the ratio of VEGF_{165b}:VEGF_{total} for 44 samples from patients treated with FOLFOX + bevacizumab (arm A) and 53 samples from patients treated with FOLFOX4 (arm B), and PFS, and overall survival (OS) analyzed on the basis of PI relative to median ratio.

Results: Unadjusted analysis of PFS showed significantly better outcome for individuals with VEGF_{165b}:VEGF_{total} ratio scores below median treated with FOLFOX4 + bevacizumab compared with FOLFOX4 alone (median, 8.0 vs. 5.2 months; $P < 0.02$), but no effect of bevacizumab on PFS in patients with VEGF_{165b}:VEGF_{total} ratio >median (5.9 vs. 6.3 months). These findings held after adjustment for other clinical and demographic features. OS was increased in arm A (median, 13.6 months) compared with arm B (10.6 months) in the low VEGF_{165b} group, but this did not reach statistical significance. There was no difference in the high VEGF_{165b}:VEGF_{total} group between FOLFOX + bevacizumab (10.8 months) and FOLFOX alone (11.3 months).

Conclusion: Low VEGF_{165b}:VEGF_{total} ratio may be a predictive marker for bevacizumab in metastatic colorectal cancer, and individuals with high relative levels may not benefit. *Clin Cancer Res*; 18(22); 6384–91. ©2012 AACR.

Introduction

Bevacizumab, a recombinant humanized monoclonal antibody that binds VEGF, has become a standard component of the treatment of patients with metastatic colorectal cancer. In 2004, a landmark study comparing bolus irinotecan, 5-fluorouracil, and leucovorin (IFL) and placebo

treatment with IFL and bevacizumab (1) showed a 4.7- and 4.2-month increase in median overall survival (OS) and progression-free survival (PFS), respectively. In 2007, the ECOG E3200 trial of 829 patients with previously treated metastatic colorectal cancer showed that addition of bevacizumab to oxaliplatin, 5-fluorouracil, and leucovorin (FOLFOX4; ref. 2) resulted in a 2.1- and 2.6-month increase in median OS and PFS, respectively. There have as yet been no definitive studies identifying biomarkers that predict outcome to bevacizumab therapy.

VEGF-A is a product of one gene encoding multiple isoforms, identified by their amino acid number and c-terminal sequence. Alternative splicing of exon 8 results in 2 families of proteins (3). Proximal splice site (PSS) usage generates a short open reading frame (ORF) of 6 amino acids common to the pro-angiogenic VEGF isoforms. An alternative distal splice site (DSS) in exon 8 results in a different 6 amino acid ORF, resulting in proteins of the same length as the pro-angiogenic isoforms, but with a different c-terminal sequence. These isoforms that are anti-angiogenic in physiologic systems (4), in experimental VEGF-driven angiogenesis (5), and in pathologic angiogenesis driven by

Authors' Affiliations: ¹Microvascular Research Laboratories, Department of Physiology and Pharmacology, University of Bristol; ²Department of Pathology, Bristol Royal Infirmary, Bristol, United Kingdom; ³Dana Farber Cancer Institute, Harvard School of Public Health, Boston, Massachusetts; ⁴University of Pennsylvania, Philadelphia, Pennsylvania; ⁵University Hospitals Seidman Cancer Center, Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio; and ⁶Northwestern University, Chicago, Illinois

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Corresponding Author: David O. Bates, Microvascular Research Laboratories, Bristol Heart Institute, Department of Physiology and Pharmacology, University of Bristol, School of Veterinary Sciences, Southwell Street, Bristol BS2 8EJ, United Kingdom. Phone: 44 117-9289818; Fax 44 117-9288151; E-mail: Dave.Bates@bristol.ac.uk

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Translational Relevance

Bevacizumab, a recombinant humanized monoclonal antibody that binds VEGF, has become a standard component of the treatment of patients with metastatic colorectal cancer. The endogenous VEGFA splice variant, VEGF_{165b}, has been shown to have anti-angiogenic properties in animal models and it binds to bevacizumab. This study of tumor samples on the original E3200 clinical trial of FOLFOX with or without bevacizumab found that only patients with a lower than median expression of VEGF_{165b} as a proportion of total VEGF benefited from treatment with bevacizumab. These results suggest that a low VEGF_{165b}:VEGF_{total} ratio may be a predictive marker for bevacizumab in metastatic colorectal cancer and individuals with high relative levels may not benefit.

VEGF (6, 7), including in tumors (5, 8–12). These isoforms, the most common of which is VEGF_{165b}, form a substantial portion of total VEGF in many tissues including normal colon (12). VEGF_{165b} is relatively downregulated in colon cancer but to a variable extent (12). VEGF_{165b} binds bevacizumab with the same affinity as VEGF₁₆₅ (12), and the overexpression of VEGF_{165b} in human colon carcinomas grown in mice conferred resistance to bevacizumab treatment (12). These findings suggest that VEGF_{165b} may interfere with bevacizumab treatment. This led us to propose that bevacizumab may be more effective against tumors in which VEGF_{165b} levels are low. We sought to test this hypothesis in samples from patients treated with bevacizumab + FOLFOX in one of the original pivotal phase III clinical trials of bevacizumab + FOLFOX in colorectal carcinoma.

Materials and Methods

Patient characteristics

In the clinical trial E3200, eligible patients were randomly assigned to receive FOLFOX4 in combination with bevacizumab (arm A); FOLFOX4 without bevacizumab (arm B); or bevacizumab alone (arm C). Random assignment was stratified on the basis of prior radiation therapy and Eastern Cooperative Group (ECOG) performance status. The primary endpoint of the study was OS. A total of 829 patients were enrolled onto the study between November 2001 and April 2003 from 221 sites in the United States and South Africa. Nine patients who received treatment were determined to be ineligible, leaving 820 randomized patients analyzable. In February 2003, the bevacizumab-alone arm of the study closed to accrual after interim analysis suggested inferior survival when compared with the chemotherapy-containing arms of the study. The final study sample sizes were therefore 286 patients (arm A), 291 patients (arm B), and 243 patients (arm C; see Fig. 1 for breakdown of cases). Human studies were conducted in accordance with the

Helsinki Declaration, and expression studies were conducted with local ethics committee approval. Tumor samples were available from 300 patients enrolled in the trial.

Immunohistochemistry

Sections (5 μ m) were cut from all 300 blinded paraffin-embedded tissue blocks taken from participants in E3200 (2). All isoforms of VEGF, termed VEGF_{total}, were detected using a commercially available anti-VEGF antibody (Dako, mouse IgG1, clone VG1). This antibody detects isoforms generated by PSS selection in exon 8 (VEGF₁₆₅, and other isoforms expressed at lower levels such as VEGF₁₂₁, VEGF₁₈₉, and others, generically termed VEGF_{xxx}) and those generated by DSS selection (VEGF_{165b}, VEGF_{121b}, VEGF_{189b}, and possibly others, generically termed VEGF_{xxx}b; refs. 5, 13). VEGF_{xxx}b expression was examined by immunohistochemistry using a mouse monoclonal IgG1 antibody raised to the terminal nine amino acids of the VEGF_{165b} sequence. It was affinity purified against the antigen from conditioned media of hybridoma cells (Abcam 14994). It has previously been shown to have specificity for the VEGF_{165b} isoform over the VEGF₁₆₅ isoforms and does not detect VEGF₁₆₅, or VEGF₁₂₁, recombinant protein (5, 13). This antibody has a VEGF_{165b} association constant of 3×10^4 (mol/L)⁻¹s⁻¹ and dissociation constant of 0.011s⁻¹ but no affinity with VEGF₁₆₅ (8). It also detects other VEGF_{xxx}b isoforms such as VEGF_{121b}, VEGF_{145b}, and VEGF_{189b} in human tissues (13). Sections were dewaxed with xylene for 15 minutes before rehydration in graded alcohols and antigen retrieved by microwave treatment for 12 minutes at 800 W in 0.01 mol/L sodium citrate buffer, pH 6.0, with the solution maintained just below the boiling point (96°C \pm 2°C). Sections were immersed in 3% hydrogen peroxide for 5 minutes and incubated with 1.5% normal horse serum (NHS) for 30 minutes in a humid chamber at room temperature. After washing, each slide was subject to incubation with 5 μ g/mL VEGF_{total} antibody in NHS or 12 μ g/mL anti-VEGF_{165b} monoclonal antibody diluted in PBS/T-1% bovine serum albumin (BSA). Sections were incubated overnight at 4°C. The blocking step with NHS was repeated before the addition of 2 μ g/mL (for VEGF_{165b}) or 2 μ g/mL (VEGF_{total}) biotinylated horse anti-mouse secondary antibody (Vector BA-2000, in NHS, 1:66 in PBS) for 30 minutes. The negative control received a matched concentration of normal mouse immunoglobulin diluted in PBS/T-1% BSA. Avidin-biotinylated enzyme complex was prepared according to the manufacturer's instructions (Elite, Vector Laboratories) and incubated with sections for 45 minutes at room temperature. This was followed by the addition of the visualizing 3,3'-diaminobenzidine (DAB) substrate for all sections and counterstaining in hematoxylin. Sections were dehydrated in graded alcohols (70%, 90%, and 100% ethanol) before final clearing in xylene and mounting with DPX and glass coverslips. Slides were dried overnight before light microscopy examination.

Slides were independently assessed and staining intensity scored (1–6). All sections were examined, conducted by 2

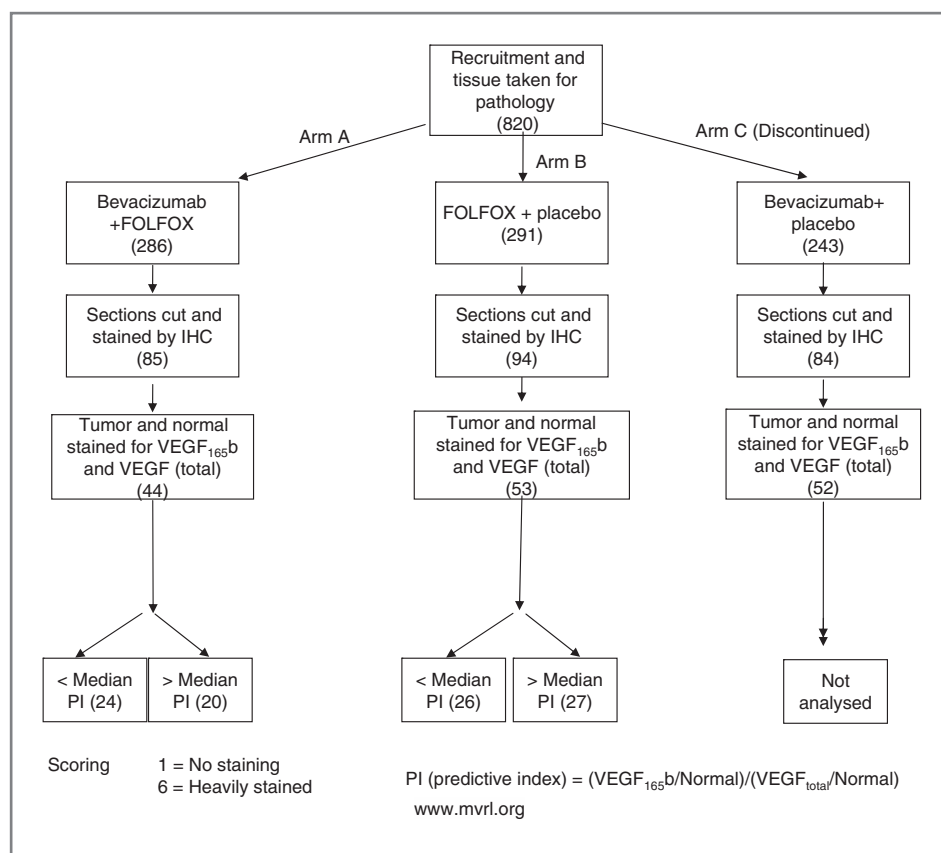


Figure 1. Breakdown of cases in the trial. IHC, immunohistochemistry.

different assessors (D.O. Bates and A.H.R. Varey), blinded to treatment. A third assessor (P. Ramani) was used to assess anomalies. They graded the intensity of DAB staining (1–6) in the normal mucosal tissue and the most poorly differentiated tumor for each section. Stroma was not scored. A score of 1 indicated no staining; 2, weak staining; 3, clear staining; 4, robust staining; 5, strong staining; and 6, intense staining (see Supplementary Figure for examples). To determine the relative intensity of the VEGF isoforms, the staining intensity in the tumor on a scale of 1 to 6 was normalized to that in the peritumoral (histologically normal) colonic mucosa for each stain. The ratio of the 2 scores was then calculated ($VEGF_{165b}:VEGF_{total}$). Thus, to obtain a score, staining was required in the normal mucosal tissue for both $VEGF_{165b}$ and $VEGF_{total}$ antibody staining. The normal mucosal tissue acted as an internal control for both antibody stains. Scores were sent to the ECOG statistical service (P.J. Catalano) for subsequent analysis. A median split was conducted to separate the samples into 2 groups—high and low $VEGF_{165b}:VEGF_{total}$. Kaplan–Meier survival curves were calculated for each of the 2 groups. Thus, the actual value of the ratio of $VEGF_{165b}:VEGF_{total}$ is reduced to a discrete value of greater or less than median. From the 300 samples in the original study, 149 were suitable for use in the final analysis, as they contained both normal mucosa and tumor and successfully stained with both $VEGF_{165b}$ and $VEGF_{total}$ antibodies, as determined by positive staining in the normal

mucosal tissue on the slides. However, 52 of these were in arm C and thus were not included in the analysis. Representative images are given in Fig. 2.

Statistical analysis

Kaplan–Meier methodology was used to present all survival curves and estimate median PFS and OS; standard proportional hazards regression modeling for censored time to event data was used to evaluate the unadjusted and adjusted relationship between $VEGF_{165b}:VEGF_{total}$ isoform ratio and outcome (PFS and OS). As of this analysis, only 11% of the cases are censored for PFS and less than 4% of the cases are censored for OS. All quoted *P* values are 2-sided, and *P* values less than 0.05 were considered statistically significant. *A priori* power analysis showed that with all 175 cases in each arm, an 83% power to detect an interaction effect (ratio of HRs of about 1.8) would be achieved for the PFS endpoint. However, an interaction effect would not be expected to be that large in the OS endpoint because the overall treatment differences were smaller in the clinical study for OS, and the OS power is therefore more limited, only 47% for an interaction effect (ratio of HRs of 1.5). Thus, the study aimed to test the hypothesis that PFS was increased. Thus, the *a priori* cutoff point for the ratio was the median value of $VEGF_{165b}$ (tumor/normal): $VEGF_{total}$ (tumor/normal). The primary endpoint of this analysis was PFS.

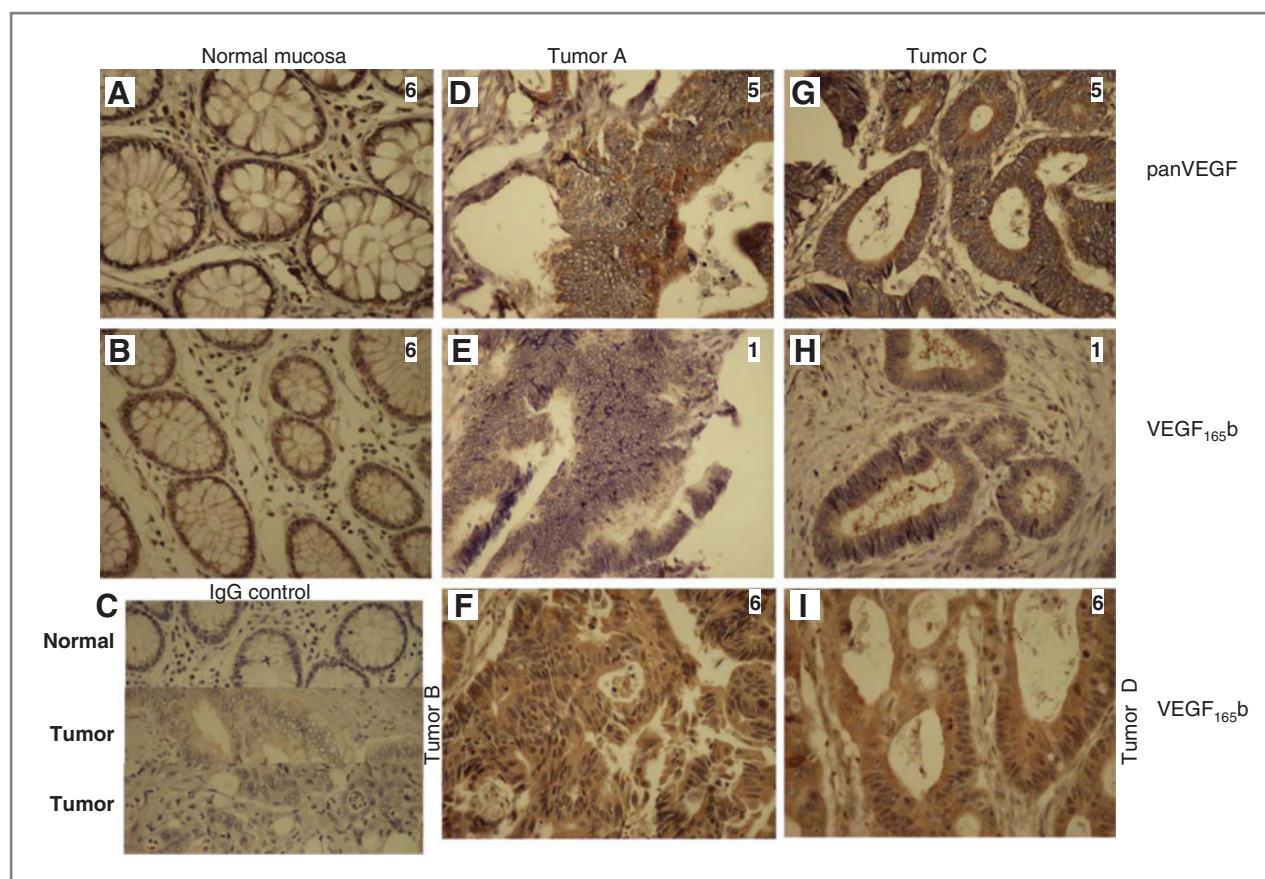


Figure 2. Examples of immunohistochemical staining for VEGF_{165b} and VEGF_{total}. Normal tissue staining for VEGF_{total} (A) and VEGF_{165b} (B) and negative IgG control (C) for normal and tumor tissue from 2 different tumors. Staining of a tumor for VEGF_{total} (D) and VEGF_{165b} (E) shows that in this tumor, there was strong expression of VEGF_{total} (scored 5) but not VEGF_{165b} (scored 1). A different tumor (F) with similar differentiation status stained heavily for VEGF_{165b} (scored 6). A third, more differentiated tumor (G) also scored heavily for VEGF_{total} (scored 5) but not VEGF_{165b} (scored 1; H). An example of a fourth tumor, with similar differentiation state heavily expressing VEGF_{165b} (I) is also shown. Numbers given are the score for each sample.

Results

Table 1 shows the breakdown of cases by treatment arm. A total of 820 cases were included in the original E3200 clinical dataset, hence 671 clinical cases were not in the VEGF isoform ratio analysis and 149 cases were included in the VEGF_{165b}:VEGF_{total} isoform ratio analysis.

There were no appreciable differences in treatment arm, sex, age, race, performance status, and the number of metastatic sites at study entry for the study overall compared with those samples included in the VEGF_{165b}:VEGF_{total} isoform ratio analyses (see Supplementary Table S1).

Figure 2 shows representative samples of tissue stained for VEGF_{165b} or VEGF_{total} from normal tissue and 2 stages of

differentiation (poor and well) of tumor. Figure 3A shows the distribution of ratios for all samples. The median score was 1.0, indicating that the VEGF_{165b} staining relative to the VEGF_{total} staining varied around equivalence. Figure 3B shows the distribution of scores. Table 2 shows the results of proportional hazards regression modeling of PFS and OS by VEGF_{165b}:VEGF_{total} isoform ratio (predictive index or PI) in tumors, for both unadjusted and adjusted analyses where the adjustment in the models included the following demographic and clinical on-study variables: sex, race, age as a continuous predictor, performance status, and the number of metastatic sites as a continuous predictor. Formal tests for the interaction between low/high VEGF_{165b}:VEGF_{total} isoform ratio and treatment arm were conducted using the proportional hazards regression model. The interaction test of the ratio by arm was significant ($P = 0.024$) for PFS but did not attain statistical significance ($P = 0.18$) for OS. These effects were also present after adjustment for demographic and clinical variables (listed above, $P_{\text{interaction}} = 0.072$).

Table 2 shows that in unadjusted analyses, significant treatment differences were observed between arm A

Table 1. Accounting of cases

Total analyzable E3200 clinical cases	820
Total VEGF _{165b} :VEGF _{total} isoform cases	149
Breakdown by treatment arm	A: 44 B: 53 C: 52

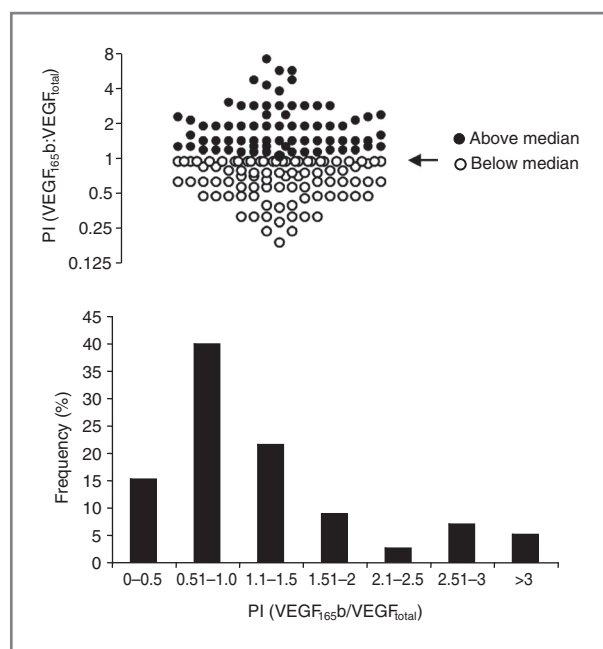


Figure 3. Distribution of PI values. A, values according to PI, with less than median VEGF_{165b} in filled symbols, and greater than median PI in open symbols. B, frequency distribution for PI values.

(bevacizumab + FOLFOX4) and arm B (FOLFOX alone) for PFS ($P = 0.018$) among patients with a low VEGF_{165b}:VEGF_{total} isoform ratio (Fig. 4A). In contrast, among patients with a high VEGF_{165b}:VEGF_{total} isoform ratio, there was no significant treatment difference between the 2 arms ($P = 0.40$ Fig. 4B). Although the HRs were in the same direction, no significant associations were observed for the OS endpoint (Fig. 4C and D).

The response rate [partial response (PR) or complete response (CR)] in the low VEGF_{165b}:VEGF_{total} isoform ratio group treated with bevacizumab + FOLFOX was 20.8% but 7.7% in those treated with FOLFOX4 alone. This compares with a response rate of 5.0% in the bevacizumab + FOLFOX-treated group with high VEGF_{165b}:VEGF_{total} isoform ratio and 7.4% in the FOLFOX4 alone-treated high VEGF_{165b}:VEGF_{total} isoform ratio group. The study was not

powered to detect a difference in response rate, and the difference was not significant by Fisher exact test.

Discussion

As the clinical use, expense and adverse effect profile of bevacizumab (and other anti-angiogenic agents such as sunitinib, sorafenib, pazopanib) has grown, the need to identify predictive markers to guide the clinical use of these agents has gained importance and urgency. Initial studies of the phase III clinical trials of bevacizumab showed no predictive value for total VEGF expression or microvessel density (14), suggesting that it was not the VEGF levels that determined outcome. Correlations between outcomes with bevacizumab and single-nucleotide polymorphisms (SNP) of VEGF in breast cancer (15); ICAM, interleukin (IL)8, and VEGF in non-small cell lung cancer (16); VEGFR1 in pancreatic and renal carcinoma (17); and hypertension in non-small cell lung cancer (18); and between VEGF SNPs and hypertension in patients with metastatic breast cancer (15) have been shown. In colonic carcinoma, low-serum angiopoietin-2 (Ang-2), an inhibitory ligand of endothelial Tie-2 receptor, has been proposed as a possible predictive marker (19), and in gastric cancer, a nonsignificant trend was seen in patients with high baseline serum VEGF (20). In breast cancer, a retrospective analysis of the mRNA or protein expression of 11 vasoactive or cell-cycle-related factors found that the low expression of delta-like ligand (Dll) 4, VEGF-C, and neuropilin-1 may identify bevacizumab-sensitive patients, although statistical significance was lost following correction for multiple hypothesis testing (21).

On the basis of evidence that the VEGF-A isoform splice variant VEGF_{165b} shows anti-angiogenic properties in animal systems (12), we postulated that patients with colorectal cancer with tumors expressing low VEGF_{165b} levels would be more likely to benefit from bevacizumab + FOLFOX-based therapy. Our findings suggest that a low VEGF_{165b}:total VEGF ratio may be a marker for clinical effect from bevacizumab + FOLFOX-based therapy.

There are a number of interpretations of the mechanisms underlying this finding. We previously showed that bevacizumab binds VEGF_{165b} with similar affinity to VEGF₁₆₅

Table 2. Summary

PI = VEGF _{165b} :VEGF _{total} isoform ratio	OS		PFS	
	HR	P	HR	P
Arm A vs. B (low PI)				
Unadjusted	0.80	0.43	0.47 (0.26–0.88)	0.018
Adjusted ^a	0.68	0.20	0.49 (0.26–0.93)	0.029
Arm A vs. B (high PI)				
Unadjusted	1.40	0.26	1.31 (0.70–2.49)	0.40
Adjusted ^a	1.05	0.89	1.19 (0.58–2.46)	0.64

^aAdjusted for race, age, performance status, and the number of metastatic sites.

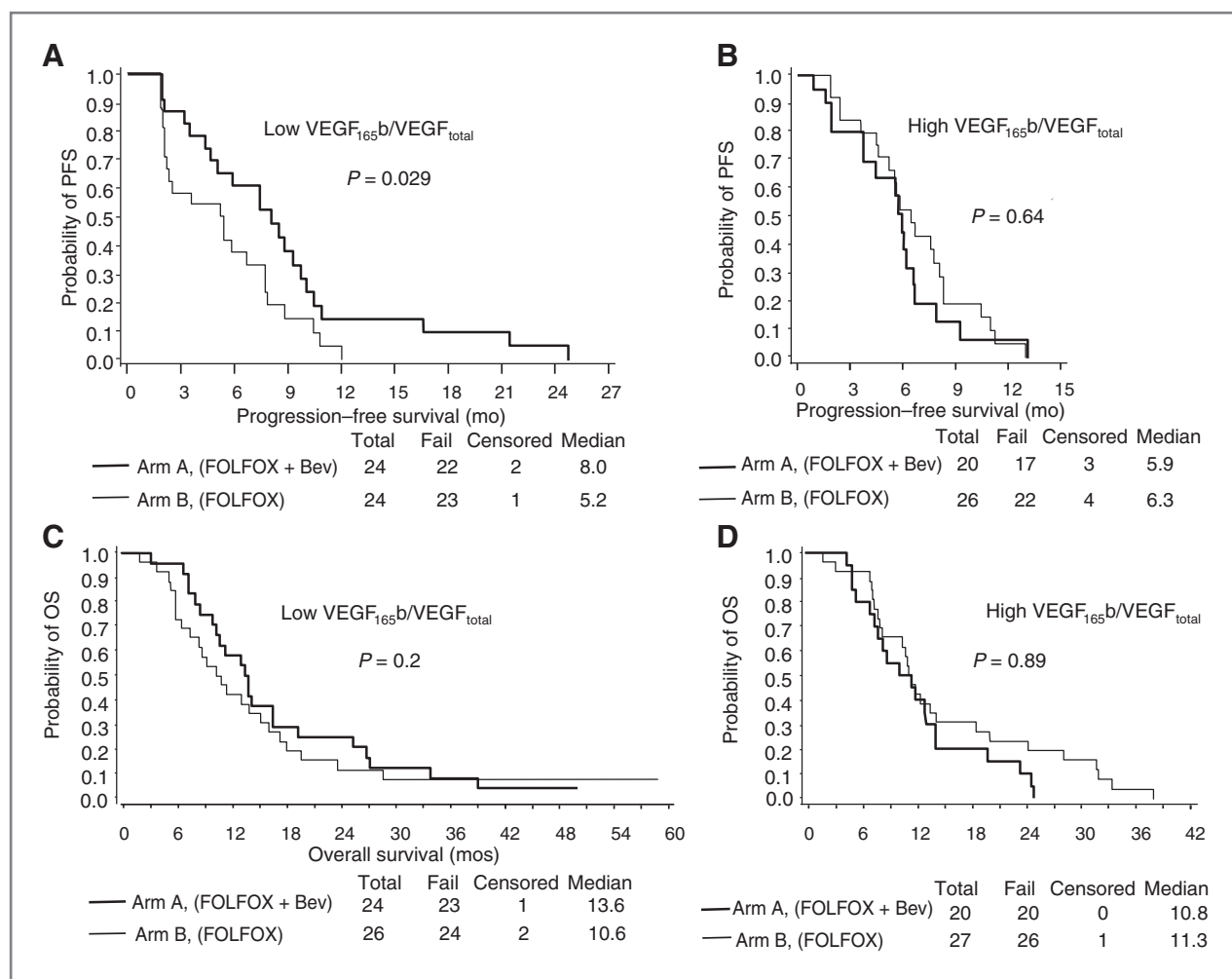


Figure 4. PFS curves for patients treated with bevacizumab (Bev) and FOLFOX4 (arm A) compared with FOLFOX4-treated patients (arm B) for high versus low relative VEGF_{165b} levels in poorly differentiated tissues. A, Kaplan–Meier curve for those patients with less than median VEGF_{165b}:VEGF_{total} ratios. B, Kaplan–Meier curve for those patients with a higher than median ratio. C, OS curves for patients treated with bevacizumab and FOLFOX4 (arm A) compared with FOLFOX4-treated patients (arm B) for high versus low relative VEGF_{165b} levels in poorly differentiated tissues. D, Kaplan–Meier curve of OS for those patients with less than median VEGF_{165b}:VEGF_{total} ratios.

(12). It is possible that in patients with a higher VEGF_{165b} level, the effective dose of bevacizumab in the tumor is lowered, as quantitatively more of the antibody is bound to VEGF_{165b} and not VEGF₁₆₅, thereby diminishing the anti-angiogenic effect of VEGF_{165b}. However, the concentration of therapeutic antibody is thought to be in excess of the VEGF levels. Conversely, patients with lower VEGF_{165b} levels may have a more angiogenic tumor, which would in turn allow for more rapid progression. Thus, a low VEGF_{165b} level could render the tumor more susceptible to bevacizumab + FOLFOX than the high VEGF_{165b} group, as angiogenesis is more likely to facilitate the progression. Finally, it is possible that binding VEGF_{165b} negates a more dominant anti-angiogenic effect than can be achieved by inhibiting the pro-angiogenic effect of VEGF₁₆₅ (12, 22, 23).

There are a number of limitations of our study. First, despite starting with a large number of samples (300), only

97 were able to be used for all 4 staining markers. Thus, the ability to detect meaningful differences in all clinically relevant endpoints, such as OS, was reduced. In addition, a larger cohort would have allowed for evaluation of the predictive function of even lower quartiles of the VEGF_{165b}:VEGF_{total} ratio. Second, the use of an immunohistochemical scoring system is subjective, and future validation using an automated quantitative system should be considered. mRNA studies would be more quantitative, although quantitative PCR for VEGF_{165b} needs to be undertaken with great care to avoid artifactual mispriming and amplification of VEGF₁₆₅. It is necessary to use appropriate controls to ensure specificity, the lack of which has caused misinterpretation of negative data in some recent studies (24). Third, our findings are in previously treated patients with advanced colorectal cancer and may not necessarily extrapolate to other tumors. And, finally, given the complexity of angiogenesis, it is unlikely that one marker will serve a

uniformly predictive function across disease types and their respective stages.

The relationship between low VEGF_{165b}:VEGF_{total} ratio and PFS in patients treated with bevacizumab + FOLFOX in E3200 supports its further evaluation as a potential biomarker and provides new insight into the tumor angiogenic network.

Disclosure of Potential Conflicts of Interest

This work is original and has not previously been published. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute. B.J. Giantonio has received honoraria from Roche and Genentech, ABB research funding and consultancy from Genentech. P.J. O'Dwyer has received research support from Genentech. N.J. Meropol received consulting fees from Precision Therapeutics. D.O. Bates and S.J. Harper are inventors on the patent describing VEGF_{165b} and use of antibodies, including as biomarkers. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.O. Bates, A.H. Varey, P.J. O'Dwyer

Development of methodology: D.O. Bates, P.J. O'Dwyer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.O. Bates, K.E. Symonds, A.H. Varey, P. Ramani, P.J. O'Dwyer, B.J. Giantonio, N.J. Meropol, A.B. Benson

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