Original Study

Angiogenic and Antiangiogenic VEGFA Splice Variants in Colorectal Cancer: Prospective Retrospective Cohort Study in Patients Treated With Irinotecan-Based Chemotherapy and Bevacizumab

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Abstract

We investigated the predictive and prognostic significance of tumoral messenger RNA levels of vascular endothelial growth factor A (VEGFS) splice variants in metastatic colorectal cancer (mCRC) patients treated with bevacizumab. VEGFA145b had negative predictive significance predominantly in those patients with right-sided primary tumors. All VEGFAxxxb variants were negative prognosticators for patients with right-sided mCRC, whereas VEGFA165b was of favorable prognostic significance in patients with left-sided tumors.

Background: Alternative splicing of vascular endothelial growth factor A (VEGFA) results in VEGFAxxxb anti-angiogenic isoforms that fail to activate angiogenesis. Bevacizumab, widely used in patients with metastatic colorectal cancer (CRC), binds both VEGFA and VEGFAxxxb isoforms. **Patients and Methods:** Formalin-fixed, paraffin-embedded primary tumors from metastatic CRC patients treated with first-line FOLFIRI (leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin) + bevacizumab (n = 285) or FOLFIRI only (n = 75) were collected. The relative expression of VEGFA121a, 121b, 145a, 145b, 165a, and 165b was assessed with custom TaqMan-MGB assays and quantitative PCR. **Results:** At a median follow-up of 101.5 months, left-sided primary CRC was a favorable prognosticator (median survival, 29.2 vs. 18.2 months; $P = .015$). Positive high VEGFA145b was an unfavorable factor for progression-free

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survival (PFS; hazard ratio [HR] = 1.66; 95% confidence interval [CI], 1.13-2.44; $P = .009$) in patients who received FOLFIRI + bevacizumab, without prognostic significance in FOLFIRI-only patients (HR = 0.70; 95% CI, 0.34-1.44; $P = .33$). The adverse effect on PFS of 145b was more pronounced in patients with right-sided colon cancer (HR = 2.62; 95% CI, 1.35-5.12; $P = .005$), especially in those who received bevacizumab (HR = 2.85; 95% CI, 1.31-6.21; $P = .008$). In patients with right-sided colon primary tumors, isoform 121b correlated with inferior PFS (HR = 1.73; 95% CI, 0.94-3.18; $P = .076$) and overall survival (OS; HR = 2.0; 95% CI, 1.08-3.72; $P = .028$). In patients with left-sided primary tumors, positive high 165b correlated with superior PFS (HR = 0.76; 95% CI, 0.59-0.99; $P = .044$) and OS (HR = 0.68; 95% CI, 0.52-0.90; $P = .006$). At multivariate analysis, right-sided primary tumor was associated with inferior PFS (HR = 1.28; 95% CI, 1.00-1.64), while 145b consistently retained predictive significance for lack of benefit in PFS with bevacizumab (HR = 1.71; 95% CI, 1.16-2.53). Multivariate analysis for OS showed that VEGFA165b expression was favorable in patients with left-sided but unfavorable in patients with right-sided primary tumors ($P_{interaction} < .001$). **Conclusion:** The antiangiogenic isoform VEGFA145b messenger RNA may predict resistance to bevacizumab. Differences in biological relevance and prognostic significance of various VEGFA isoforms were found for right- versus left-sided primary tumors.

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Introduction

Angiogenesis, the formation of new blood vessels from preexisting ones, is an essential component of tumor development. Several strategies to modulate angiogenesis have been developed so far, wit antibodies against vascular endothelial growth factor A (VEGFA) and tyrosine kinase inhibitors being the most representative agents.[1] Antiangiogenic treatment response varies among cancer patients. Resistance can develop during the course of treatment as a result of redundancy of alternative angiogenic pathways that compensate for VEGFA blockade and through metabolic reprogramming. In addition to acquired resistance, certain tumors may exhibit intrinsic unresponsiveness to vascular endothelial growth factor (VEGF)-targeted therapies.[2] Possible mechanisms include the ability of tumor cells to co-opt the normal vasculature of the host tissue, the involvement of VEGF-independent mechanisms of angiogenesis, and the property of certain tumor cells to survive in a hostile hypoxic microenvironment. The presence of angiogenic cascades less amenable to VEGFA inhibition has also been suggested.[3,4] Consequently, the discovery of predictive biomarkers is vital to identify patients who will benefit from antiangiogenic therapy. Although imaging and functional vascular parameters have been proposed as potential predictive biomarkers, they have not been validated in large groups of patients; nor has the pretreatment levels of certain angiogenic proteins, or the circulating endothelial cell levels or plasma levels of VEGF and vascular endothelial growth factor receptor 2 (VEGFR2).[5] It is thus evident that surrogate biomarkers predicting response to anti-VEGF targeting agents are still missing.

Although VEGFA is a major angiogenic growth factor, several of its isoforms exert antiangiogenic actions.[6] VEGFA pre—messenger RNA (mRNA) is subject to alternative splicing that leads to the generation of distinct VEGFAxxx isoforms, where xxx denotes the number of amino acids in the protein sequence. In addition to the variable exonic composition, VEGFA isoforms differ also in the sequence of exon 8. Differential splicing in the 3′ proximal versus in the 3′ distal site of exon 8 results in the VEGFAxxxa and VEGFAxxxb subclasses with proangiogenic and antiangiogenic roles, respectively. The balance between VEGFAxxxa and VEGFAxxxb may regulate the angiogenic potential of a malignancy, with investigators reporting varying ratios of angiogenic to antiangiogenic VEGFA isoforms in tumors (ranging from 1:1 to 20:1).[7]

Bevacizumab is a recombinant humanized monoclonal immunoglobulin G1 antibody that blocks VEGFA and plays a central role in the management of metastatic colorectal cancer (CRC) patients. Bevacizumab has the ability to bind and sequester both VEGFAxxxa and VEGFAxxxb isoforms. These properties may be counteracting and may account for the lack of response in cases where VEGFAxxxb expression is high.[8] Nevertheless, to date, tumor VEGFAxxxb levels have not been adequately investigated as a biomarker of bevacizumab benefit.

Considering the lack of validated predictive biomarkers for bevacizumab and the underlying biological hypothesis of a role of VEGFAxxxb antiangiogenic isoforms in the regulation of malignant angiogenesis, we sought to investigate the prognostic and predictive significance of VEGFA121a, 121b, 145a, 145b, 165a, and 165b tumoral mRNA levels in patients with metastatic CRC (mCRC).

Patients and Methods

All patients had a histologic diagnosis of colorectal adenocarcinoma from primary or metastatic lesions, were prospectively accrued in a Hellenic Cooperative Oncology Group (HeCOG) registry in affiliated centers from 2003 to 2010, and were managed with first-line standard irinotecan and fluoropyrimidine-based (FOLFIRI [leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin] or CapIRI [capecitabine, irinotecan]) protocols with bevacizumab (n = 285) or without targeted agents (n = 75). Systemic therapy was selected on the basis of the physician's choice, taking into consideration individual disease demographics as well as patient comorbidity and preferences. Formalin-fixed, paraffin-embedded (FFPE) tumor blocks from the primary or metastatic tumor were prospectively collected and retrospectively analyzed for mRNA expression of VEGFA splice variants. Clinicopathologic data were prospectively collected for all patients in a Web-based electronic database in the

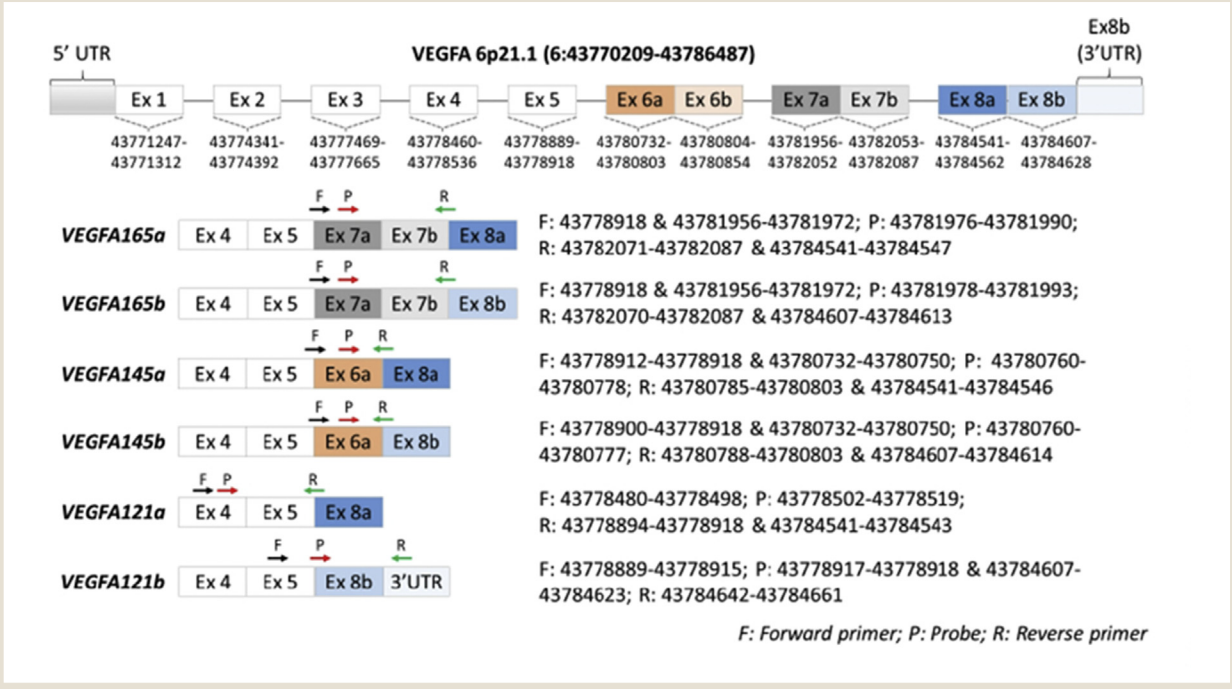
context of the registry. All patients provided written informed consent for research use of their biological material.

The translational research protocol was conducted in agreement with the Declaration of Helsinki and was approved by the institutional review boards of "Papageorgiou" Hospital (1337/12-1-2015) and "Thermi" Clinic (307/2-3-2016). End points of the retrospective analysis were 3-fold: (1) study the distribution of VEGFA isoforms in the tumor, both angiogenic and antiangiogenic, (2) examine their associations with clinicopathologic characteristics (including tumor location and *KRAS* mutation) and VEGFA splice variants, and (3) examine their prognostic and predictive significance for outcome and benefit of bevacizumab, respectively.

Molecular Analysis

Assessment of VEGFA Splice Variants. We processed FFPE sample blocks from 391 patients, yielding evaluable RNA in 360. Hematoxylin and eosin–stained sections were reviewed by an expert pathologist who marked tumor-dense areas and assessed tumor cell content. After manual macrodissection from 10 μm unstained sections, tissue fragments were processed for overnight lysis at 56°C in the presence of 500 μg/mL proteinase K. Total RNA was isolated from tissue lysates with TRIzol-LS (Invitrogen; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions; 4 to 5 μg of total RNA were reverse transcribed with random hexamers and SuperScript III Reverse Transcriptase, according to standard procedures (cat. nos. 48190011 and 18080044, respectively; Invitrogen). We interrogated the expression of VEGFA mRNA isoforms with quantitative PCR (qPCR). On the basis of previous literature[7,9] and data from the National Center for Biotechnology Information, we designed proangiogenic (xxxa) and antiangiogenic (xxxb) VEGFA mRNA transcripts with qPCR and custom, splice-variant specific assays, spanning exons 5-7a and 8a or 8b for VEGFA165a or 165b; exons 5-6a and 8a or 8b for VEGFA145a or 145b; exons 5-8a or 5-8b for VEGFA121a or 121b, respectively (Figure 1). We also assessed total VEGFA mRNA expression with a premade assay (Hs00900055_m1) spanning 59 bp in the junction of exons 3-4, a region preserved in all VEGFA isoforms. As an endogenous control, we used an assay targeting β-glucuronidase (GUSB) mRNA (Hs99999908_m1). The commercially available TaqMan Control Total RNA (cat. no. 4307281, Applied Biosystems; Thermo Fisher Scientific) was applied as a positive control for interrun evaluation of PCR assay efficiency, together with no-template controls. Complementary DNA (cDNA) samples (50 ng per reaction) were ran in duplicate reactions in the 7900HT system under default conditions. To obtain linear relative quantification (RQ) values, relative expression was assessed as $(40 - \Delta C_t)$, where $\Delta C_t = (\text{avg } C_t \text{ target}) - (\text{avg } C_t$

Figure 1 **Assays for Detection of VEGFA Proangiogenic (xxxa) and Antiangiogenic (xxxb) Isoforms With Custom TaqMan qPCR Assays. Schematic at Top Shows Exon Structure of *VEGFA* Gene Along With 5′ and 3′ UTR. Exons 6, 7, and 8 Are Further Subdivided Each Into Exon "a" or "b" as Result of Alternative Splicing. Exon Structure of Individual VEGFAxxxa and -xxxb Isoforms Is Presented Schematically. Black, Red, and Green Arrows Indicate Exon Regions Spanned by Forward Primer, Hybridization Probe, and Reverse Primer of Each qPCR Assay. Respective Chromosome Coordinates are Shown at Right of Each Isoform. All Gene, Exon, and Assay Coordinates on Chromosome 6 Are According to GRCh38 Primary Assembly**



Abbreviations: qPCR = quantitative PCR; VEGFA = vascular endothelial growth factor A.

GUSB).[10] Samples were considered eligible if GUSB $C_t < 36$ and $\Delta RQ < 1$ for each duplicate pair (intrarun variation).

KRAS *and* BRAF *Mutational Analysis.* Tumor DNA was extracted with QIAamp DNA mini kit (Qiagen, Germantown, MD) upon macrodissection for enrichment in tumor cells. *KRAS* mutations at codons 12 and 13 were detected with qPCR and Sanger sequencing, as previously described.[11] For $BRAF^{V600E}$ in exon 15, we used the same approach with a qPCR allelic discrimination assay and Sanger sequencing on M13-coupled nested PCR products (amplicon coordinates [GRCh 38] on chromosome 7: 140753188-140753404).

Statistical Analysis

RQ values of < 31 corresponded to negative mRNA expression for all examined markers. For positive mRNA expression (RQ values ≥ 31), we plotted the respective distributions for all markers (Supplemental Figure 1 in the online version) in order to decide on suitable cutoffs by examining the lower and upper values of the quartiles, as well as the natural breaks in these distributions. Selected cutoffs for VEGFA121a, 121b, 145a, 165a, and 165b were the 25th percentile of the RQ distribution, while the 75th percentile was used as a cutoff for VEGFA145b in order to categorize tumors with positive mRNA expression into positive high and positive low mRNA expression.

Group comparisons of categorical data were assessed by the chi-square or Fisher exact (where appropriate) test, while Wilcoxon rank-sum tests were performed to detect differences between categorical and continuous variables. Progression-free survival (PFS) was defined as the time from diagnosis of metastatic disease to the date of documented disease progression, death, or last contact, whichever occurred first. Overall survival (OS) was also measured from the diagnosis of metastatic disease to the date of death. Alive patients were censored at the date of their last contact. Survival curves were estimated by the Kaplan-Meier method and compared across groups with the log-rank test. The associations between the factors of interest and progression/mortality rates were evaluated with hazard ratios (HR) estimated with univariate and multivariate Cox proportional hazard regression models. The reference group for mRNA expression of all markers was the group of patients with negative mRNA expression. In case the marker was of prognostic/predictive significance overall as a result of the HR associated with positive high versus positive low (rather than negative) mRNA expression, the two categories (positive low, negative) were merged because the risk of progression or death was similar in the two indicated groups.

All tests were 2 sided at a 5% level of significance. Analyses were conducted by SAS 9.3 software (SAS Institute, Cary, NC).

Results

Patient Demographics

A total of 360 mCRC patients with evaluable FFPE-derived cDNA were included in the current registry. Among them, 285 patients (79.2%) had additionally received bevacizumab, while 75 (20.8%) were administered chemotherapy only. Table 1 presents selected patient and tumor characteristics. The median age at diagnosis was 64.1 years, while more than half of the patients were men (60.8%) and had left-sided primary tumors (71.9%). Patients treated with bevacizumab were young (Wilcoxon rank-sum $P < .001$) and had a better performance status than those treated with chemotherapy (chi-square $P = .013$). Regarding molecular characterization, *KRAS* status was informative for 354 patients (98.3%), and 174 (49.2%) of them carried mutations in *KRAS*, while only 13 (4.2%) had *BRAF* mutations.

Table 1 Patient and Tumor Demographics

Characteristic	Chemotherapy and Bevacizumab (N = 285)	Chemotherapy Only (N = 75)	Total (N = 360)
Age (Y)			
Median	63.1	69.1	64.1
Interquartile range	(56.2, 69.4)	(60.2, 74.4)	(57.5, 70.7)
Gender			
Female	117 (41.1)	24 (32.0)	141 (39.2)
Male	168 (58.9)	51 (68.0)	219 (60.8)
Tumor Site			
Left colon	209 (73.3)	50 (66.7)	259 (71.9)
Right colon	75 (26.3)	24 (32.0)	99 (27.5)
Unknown	1 (0.4)	1 (1.3)	2 (0.6)
Performance Status			
0	209 (73.3)	42 (56.0)	251 (69.7)
1-2	73 (25.6)	29 (38.7)	102 (28.3)
Unknown	3 (1.1)	4 (5.3)	7 (1.9)
No. of Metastatic Sites			
1-2	258 (90.5)	59 (78.7)	317 (88.1)
≥3	19 (6.7)	9 (12.0)	28 (7.8)
Unknown	8 (2.8)	7 (9.3)	15 (4.2)
Metastatic Site			
Liver only	125 (43.9)	28 (37.3)	153 (42.5)
Nodal	37 (13.0)	8 (10.7)	45 (12.5)
Visceral	99 (34.7)	27 (36.0)	126 (35.0)
Other/unknown	24 (8.4)	12 (16.0)	36 (10.0)
***KRAS* Status**			
Mutant	137 (48.1)	37 (49.3)	174 (48.3)
Wild type	142 (49.8)	38 (50.7)	180 (50.0)
Unknown	6 (2.1)	0 (0.0)	6 (1.7)
***BRAF* Status**			
Mutant	12 (4.2)	1 (1.3)	13 (3.6)
Wild type	231 (81.1)	67 (89.3)	298 (82.8)
Unknown	42 (14.7)	7 (9.3)	49 (13.6)

Data are presented as n (%) unless otherwise indicated.

The frequency distribution of the examined markers for the entire study population, and by treatment type and tumor location is presented in Table 2. No significant associations were detected between the expression of the VEGFA splice variants and the administered therapy or the tumor location. Statistically significant

Table 2 VEGFA Splice Variant Frequency Distribution by Therapy and Tumor Location

Parameter	Category	Total	Therapy			Site		
			Chemotherapy and Bevacizumab	Chemotherapy Only	*P*	Left Colon	Right Colon	*P*
VEGFA121a mRNA expression	Negative	39 (10.8)	29 (10.2)	10 (13.3)	.28	34 (13.1)	5 (5.1)	.090
	Positive high	240 (66.7)	187 (65.6)	53 (70.7)		168 (64.9)	70 (70.7)	
	Positive low	81 (22.5)	69 (24.2)	12 (16.0)		57 (22.0)	24 (24.2)	
VEGFA121b mRNA expression	Negative	70 (19.4)	59 (20.7)	11 (14.7)	.48	51 (19.7)	18 (18.2)	.85
	Positive high	217 (60.3)	170 (59.6)	47 (62.7)		157 (60.6)	59 (59.6)	
	Positive low	73 (20.3)	56 (19.6)	17 (22.7)		51 (19.7)	22 (22.2)	
VEGFA145a mRNA expression	Negative	37 (10.3)	33 (11.6)	4 (5.3)	.28	29 (11.2)	8 (8.1)	.51
	Positive high	242 (67.2)	188 (66.0)	54 (72.0)		170 (65.6)	71 (71.7)	
	Positive low	81 (22.5)	64 (22.5)	17 (22.7)		60 (23.2)	20 (20.2)	
VEGFA145b mRNA expression	Negative	73 (20.3)	60 (21.1)	13 (17.3)	.52	53 (20.5)	20 (20.2)	.99
	Positive high	71 (19.7)	53 (18.6)	18 (24.0)		51 (19.7)	19 (19.2)	
	Positive low	216 (60.0)	172 (60.4)	44 (58.7)		155 (59.8)	60 (60.6)	
VEGFA165a mRNA expression	Negative	54 (15.0)	47 (16.5)	7 (9.3)	.24	43 (16.6)	11 (11.1)	.42
	Positive high	229 (63.6)	176 (61.8)	53 (70.7)		162 (62.5)	65 (65.7)	
	Positive low	77 (21.4)	62 (21.8)	15 (20.0)		54 (20.8)	23 (23.2)	
VEGFA165b mRNA expression	Negative	34 (9.4)	29 (10.2)	5 (6.7)	.64	24 (9.3)	10 (10.1)	.28
	Positive high	244 (67.8)	191 (67.0)	53 (70.7)		170 (65.6)	72 (72.7)	
	Positive low	82 (22.8)	65 (22.8)	17 (22.7)		65 (25.1)	17 (17.2)	

Data are presented as n (%).
Abbreviations: mRNA = messenger RNA; VEGFA = vascular endothelial growth factor A.

associations were observed between 121b, 165b, and nodal status (higher RQ in N1-2 as opposed to N0 disease; $P = .018$), -145b, and the presence of visceral metastases (higher RQ in visceral, as opposed to liver-only or nodal, disease; $P = .007$). Associations between VEGFA isoform RQ and the examined clinicopathologic characteristics are presented in Supplemental Table 1 in the online version.

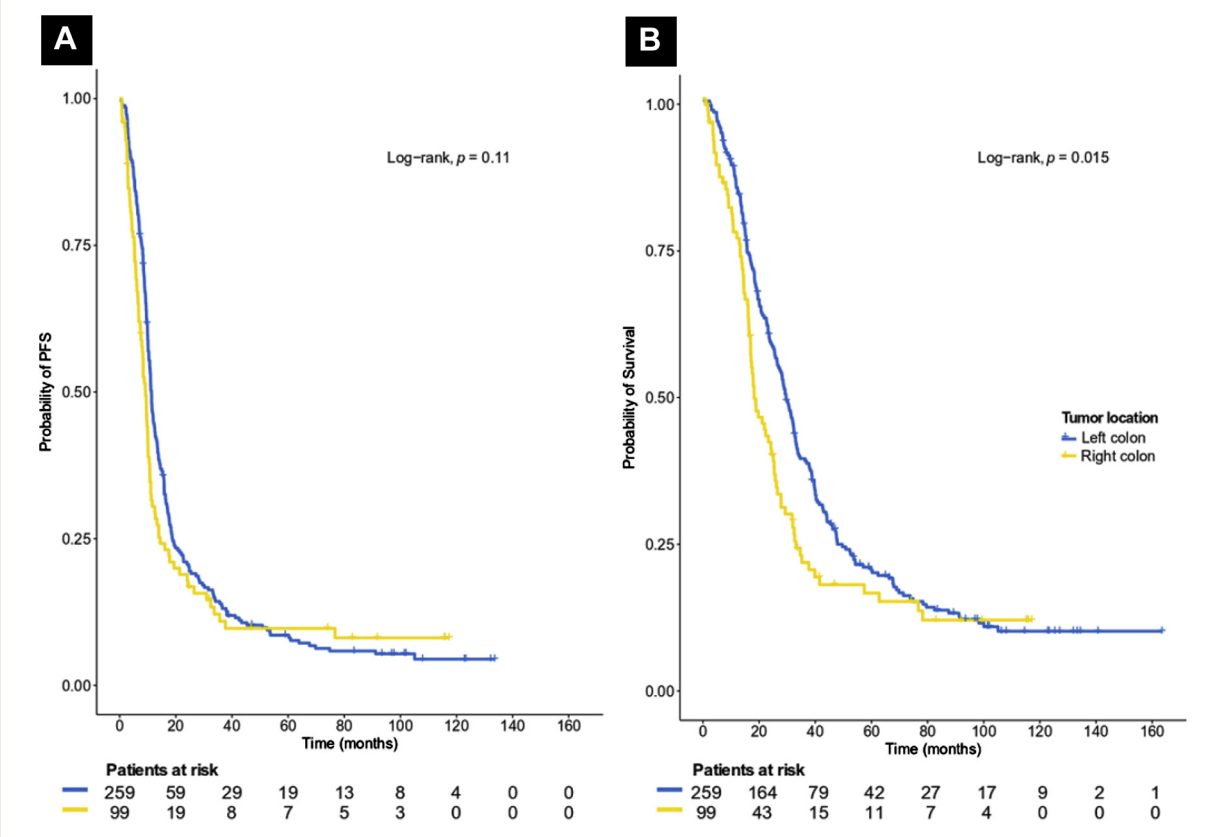
At a median follow-up of 101.5 months (95% confidence interval [CI], 93.48-115.21), a total of 301 patients (83.6%) were reported dead and 276 patients (76.7%) had experienced disease progression. The median PFS was 10.7 months (95% CI, 9.90-11.31), while the median OS was 25.8 months (95% CI, 23.28-29.21). Patients with left-sided primary tumors had superior OS compared to those with right-sided tumors (median OS, 29.2 [95% CI, 25.61-32.30] vs. 18.2 [95% CI, 16.39-24.30], log-rank $P = .015$), while no difference was observed between them in terms of PFS (log-rank $P = .11$) (Figure 2). The outcome of patients by treatment type and tumor location is summarized in Supplemental Table 2 in the online version.

Association of Tumor VEGFA Isoforms With Patient Outcome

Progression-Free Survival. A significant interaction was detected between the type of therapy and 145b with respect to PFS ($P_{interaction} = .040$). Indeed, 145b was an unfavorable factor for PFS among patients who received a regimen with bevacizumab. Specifically, patients with tumors that had positive high 145b mRNA expression exhibited an increased risk of progression compared to patients with negative 145b mRNA expression (HR = 1.66; 95% CI, 1.13-2.44, Wald $P = .009$). In contrast, 145b mRNA expression did not reach significance among patients who were not administered bevacizumab ($P_{overall} = .56$); in fact, the HR of patients with positive high 145b was in the opposite direction (HR = 0.70; 95% CI, 0.34-1.44; $P = .33$). Moreover, a significant interaction was seen between 145b and tumor location ($P_{interaction} = .007$). The adverse prognostic effect of 145b was robust in patients with right-sided primary tumors, in whom positive high 145b was associated with increased risk of progression (HR = 2.62; 95% CI, 1.35-5.12; $P = .005$), while it was lost in patients with left-sided primary tumors ($P = .43$). The increased risk of progression of patients with 145b-positive high tumors was even more marked in patients with CRC with right-sided tumors managed with bevacizumab (HR = 2.85; 95% CI, 1.31-6.21; $P = .008$), which again was not observed in patients with left-sided primary tumors ($P = .057$). Indeed, a trend was detected in bevacizumab-treated patients for the interaction of 145b mRNA expression and tumor location in terms of PFS ($P_{interaction} = .081$).

Significant interactions were also observed between tumor location and VEGFA121b and VEGFA165b with respect to PFS ($P_{interaction} = .011$ and .010, respectively). In the subgroup of

Figure 2 PFS (A) and OS (B) by Tumor Location



Abbreviations: OS = overall survival; PFS = progression-free survival.

patients with right-sided tumors, the risk of progression was higher for patients with 121b-positive mRNA expression (positive high compared to negative, HR = 1.73; 95% CI, 0.94-3.18; $P = .076$; positive low compared to negative, HR = 2.48; 95% CI, 1.23-5.04; $P = .012$). No PFS prognostic effect was seen for 121b in CRC patients with left-sided tumors ($P = .59$). In bevacizumab-treated patients, a similar significant interaction was observed between 121b mRNA expression and tumor location with respect to PFS ($P_{interaction} = .029$).

In contrast, 165b mRNA expression exhibited favorable prognostic significance in patients with left-sided tumors (positive high vs. positive low/negative 165b mRNA PFS, HR = 0.76; 95% CI, 0.59-0.99; $P = .044$). In patients with right-sided primary tumors, the HR of positive high VEGFA165b expression was in the opposite direction, with a trend toward significance for PFS (HR = 1.59; 95% CI 0.97-2.62; $P = .065$). There was no evidence of a statistically significant interaction between 165b and the type of therapy provided to patients with left-sided primary tumors ($P_{interaction} = .23$) (Tables 3 and 4).

Overall Survival. With respect to OS, significant interactions were identified between VEGFA121b, VEGFA165b, and tumor location ($P_{interaction} = .019$ and $p < 0.001$, respectively). Similar to the observed 121b adverse PFS prognostic effect in patients with CRC with right-sided tumors, those with tumors of positive high 121b mRNA expression in the right colon had worse OS compared to those with negative 121b mRNA expression (HR = 2.00; 95% CI, 1.08-3.72; $P = .028$). No significance was reached for 121b among patients with left-sided tumors ($P = .43$). In bevacizumab-treated patients, a significant interaction was observed between 121b mRNA expression and tumor location with respect to OS ($P_{interaction} = .011$). Patients who received bevacizumab and were diagnosed with right-sided tumors of positive high VEGFA121b mRNA expression had worse OS compared to patients with right-sided primary tumors and negative 121b mRNA expression (HR = 2.55; 95% CI, 1.22-5.34; $P = .013$).

Observations on the OS prognostic significance of VEGFA165b were also in agreement with those observed for PFS. Specifically, 165b was of favorable prognostic significance for OS in patients with left-sided tumors and of unfavorable significance among those with right-sided primary tumors. Patients with positive high (vs. positive low/negative) 165b mRNA expression and right-sided tumors yielded a HR of 1.83 (95% CI, 1.08-3.08; $P = .024$). The opposite was observed for 165b positive

Table 3 VEGFA Splice Variants With Prognostic Significance for Progression-Free Survival at Univariate Analysis

Parameter	Category	No. of Patients	No. of Events	HR	95% CI	*P*
Treatment Type, VEGFA145b mRNA Expression	$P_{interaction}$ = .040					
Beva						.003
	Positive high vs. negative	53 vs. 60	52 vs. 55	1.66	1.13-2.44	.009
	Positive low vs. negative	172 vs. 60	153 vs. 55	0.96	0.71-1.31	.81
Chemotherapy only						.56
	Positive high vs. negative	18 vs. 13	17 vs. 13	0.70	0.34-1.44	.33
	Positive low vs. negative	44 vs. 13	40 vs. 13	0.91	0.49-1.71	.77
Among patients with positive high VEGFA145b mRNA expression	Non-beva vs. beva	18 vs. 53	17 vs. 52	0.55	0.31-0.98	.042
Among patients with positive low VEGFA145b mRNA expression	Non-beva vs. beva	44 vs. 172	40 vs. 153	1.28	0.90-1.82	.17
Among patients with negative VEGFA145b mRNA expression	Non-beva vs. beva	13 vs. 60	13 vs. 55	1.34	0.73-2.47	.35
Tumor Location, VEGFA121b mRNA Expression	$P_{interaction}$ = .011					
Right-sided primary tumors						.041
	Positive high vs. negative	59 vs. 18	54 vs. 13	1.73	0.94-3.18	.076
	Positive low vs. negative	22 vs. 18	20 vs. 13	2.48	1.23-5.04	.012
Left-sided primary tumors						.59
	Positive high vs. negative	157 vs. 51	145 vs. 50	0.94	0.68-1.30	.70
	Positive low vs. negative	51 vs. 51	46 vs. 50	0.82	0.55-1.22	.32
Tumor Location, VEGFA145b mRNA Expression	$P_{interaction}$ = .007					
Right-sided primary tumors						.006
	Positive high vs. negative	19 vs. 20	19 vs. 18	2.62	1.35-5.12	.005
	Positive low vs. negative	60 vs. 20	50 vs. 18	1.16	0.68-1.99	.59
Left-sided primary tumors						.43
	Positive high vs. negative	51 vs. 53	49 vs. 50	1.08	0.73-1.61	.69
	Positive low vs. negative	155 vs. 53	142 vs. 50	0.88	0.64-1.22	.46
Tumor Location, VEGFA165b mRNA Expression	$P_{interaction}$ = .010					
Right-sided primary tumors						
	Positive high vs. positive low/negative	72 vs. 27	66 vs. 21	1.59	0.97-2.62	.065
Left-sided primary tumors						
	Positive high vs. positive low/negative	170 vs. 89	154 vs. 87	0.76	0.59-0.99	.044

Abbreviations: beva = bevacizumab; CI = confidence interval; HR = hazard ratio; mRNA = messenger RNA; VEGFA = vascular endothelial growth factor A.

high expression among patients with left-sided primary tumors (HR = 0.68; 95% CI, 0.52-0.90; P = .006). There was no evidence of a statistically significant interaction between 165b and the type of therapy provided to patients with left-sided primary tumors (Tables 4 and 5).

Bevacizumab Therapy and KRAS *Mutational Status*

The interactions of VEGFA isoforms with the *KRAS* mutational status (exon 2, 3, or 4 mutant vs. wild type) were also examined among patients treated with bevacizumab. A significant interaction was detected between VEGFA121a mRNA expression and *KRAS* mutational status in terms of PFS ($P_{interaction}$ = .037). Among bevacizumab-treated patients with mutant *KRAS*, patients with tumors of positive high VEGFA121a mRNA expression had longer PFS compared to those with negative VEGFA121a mRNA expression (HR = 0.48; 95% CI, 0.28-0.83; P = .009). All interactions of VEGFA isoforms with tumor location and *KRAS* status in the bevacizumab-treated cohort are summarized in Table 4.

Multivariate Analysis for PFS

A multivariate analysis was undertaken in the entire study population with respect to PFS, including gender (female vs. male), tumor location (right vs. left colon), treatment type (chemotherapy only vs. chemotherapy and bevacizumab), VEGFA145b mRNA expression (positive high, positive low vs.

Table 4 VEGFA Isoforms With Univariate Prognostic Significance for PFS and OS in Bevacizumab-Treated Patients

Parameter	Category	No. of Patients	No. of Events	HR	95% CI	*P*
PFS						
Tumor Location, VEGFA121b mRNA Expression	$P_{interaction}$ = .029					
Right-sided primary tumors						.067
	Positive high vs. negative	45 vs. 14	42 vs. 9	2.05	0.99-4.23	.052
	Positive low vs. negative	16 vs. 14	14 vs. 9	2.69	1.15-6.28	.023
Left-sided primary tumors						.62
	Positive high vs. negative	124 vs. 45	114 vs. 44	1.01	0.71-1.43	.97
	Positive low vs. negative	40 vs. 45	36 vs. 44	0.84	0.54-1.30	.43
Tumor Location, VEGFA145b mRNA Expression	$P_{interaction}$ = .081					
Right-sided primary tumors						.014
	Positive high vs. negative	13 vs. 17	13 vs. 15	2.85	1.31-6.21	.008
	Positive low vs. negative	45 vs. 17	37 vs. 15	1.13	0.62-2.06	.70
Left-sided primary tumors						.057
	Positive high vs. negative	40 vs. 43	39 vs. 40	1.39	0.89-2.17	.14
	Positive low vs. negative	126 vs. 43	115 vs. 40	0.89	0.62-1.28	.53
KRAS Mutational Status, VEGFA121a mRNA Expression	$P_{interaction}$ = .037					
Mutant *KRAS*						.025
	Positive high vs. negative	92 vs. 16	84 vs. 16	0.48	0.28-0.83	.009
	Positive low vs. negative	29 vs. 16	28 vs. 16	0.65	0.35-1.20	.16
Wild-type *KRAS*						.54
	Positive high vs. negative	92 vs. 13	85 vs. 12	1.36	0.74-2.49	.32
	Positive low vs. negative	37 vs. 13	29 vs. 12	1.17	0.60-2.30	.64
Overall Survival						
Tumor Location, VEGFA121b mRNA Expression	$P_{interaction}$ = .011					.030
Right-sided primary tumors	Positive high vs. negative	45 vs. 14	39 vs. 9	2.55	1.22-5.34	.013
	Positive low vs. negative	16 vs. 14	11 vs. 9	1.54	0.63-3.71	.34
Left-sided primary tumors						.65
	Positive high vs. negative	124 vs. 45	102 vs. 40	0.86	0.60-1.25	.44
	Positive low vs. negative	40 vs. 45	34 vs. 40	0.82	0.52-1.29	.39

Abbreviations: CI = confidence interval; HR = hazard ratio; mRNA = messenger RNA; OS = overall survival; PFS = progression-free survival; VEGFA = vascular endothelial growth factor A.

negative), and the interaction of VEGFA145b with treatment type (Figure 3).

VEGFA145b mRNA expression remained an unfavorable parameter for PFS among bevacizumab-treated patients, with patients with positive high VEGFA145b mRNA expression being at higher risk of progression (HR = 1.71; 95% CI, 1.16-2.53; P = .007), whereas it failed to do so in patients not exposed to bevacizumab (HR = 0.75; 95% CI, 0.36-1.58; P = .45). Among patients with positive high 145b mRNA expression, non-administration of bevacizumab was associated with arithmetically superior PFS compared to patients treated with bevacizumab (HR = 0.59; 95% CI, 0.32-1.08; P = .089).

Right-sided primary location was marginally associated with worse PFS (HR = 1.28; 95% CI, 1.00-1.64; P = .051).

Multivariate Analysis for OS

In the multivariate analysis with respect to OS in the entire study cohort, we included the aforementioned clinicopathologic parameters, VEGFA165b mRNA expression (positive high vs. positive low/negative), and the interaction of VEGFA165b mRNA expression with tumor location. A significant interaction between 165b mRNA expression and primary tumor location was confirmed ($P_{interaction}$ < .001). Specifically, in patients with right-sided primary tumors, positive high 165b was associated with increased risk of death (HR = 1.87; 95% CI, 1.10-3.17; P = .021). In contrast, patients with left-sided primary tumors and positive high 165b expression exhibited superior survival to those of positive low/negative expression (HR = 0.68; 95% CI, 0.51-0.89; P = .005) (Figure 4).

Discussion

In a retrospective study of prospectively collected FFPE CRC blocks from patients uniformly treated with first-line fluoropyrimidine–irinotecan–based chemotherapy with or without bevacizumab, we report a predictive significance of the VEGFA145b splice variant mRNA expression in tumor. In particular, patients with high mRNA levels of this isoform had worse PFS when treated with bevacizumab compared to patients expressing low levels in

Table 5 VEGFA Splice Variants With Prognostic Significance for Overall Survival at Univariate Analysis

Parameter	Category	No. of Patients	No. of Events	HR	95% CI	*P*
Tumor Location, VEGFA121b mRNA Expression	$P_{interaction} = .019$					
Right-sided primary tumors						.085
	Positive high vs. negative	59 vs. 18	51 vs. 13	2.00	1.08-3.72	.028
	Positive low vs. negative	22 vs. 18	17 vs. 13	1.60	0.78-3.30	.20
Left-sided primary tumors						.43
	Positive high vs. negative	157 vs. 51	131 vs. 46	0.82	0.58-1.14	.23
	Positive low vs. negative	51 vs. 51	42 vs. 46	0.78	0.51-1.19	.25
Tumor location, VEGFA165b mRNA Expression	$P_{interaction} = .001$					
Right-sided primary tumors						
	Positive high vs. positive low/negative	72 vs. 27	62 vs. 19	1.83	1.08-3.08	.024
Left-sided primary tumors						
	Positive high vs. positive low/negative	170 vs. 89	138 vs. 81	0.68	0.52-0.90	.006

Abbreviations: CI = confidence interval; HR = hazard ratio; mRNA = messenger RNA; VEGFA = vascular endothelial growth factor A.

their tumors, while no impact was observed in patients not exposed to bevacizumab. This is the first report implicating VEGFA145b as a negative predictive marker for bevacizumab therapy.

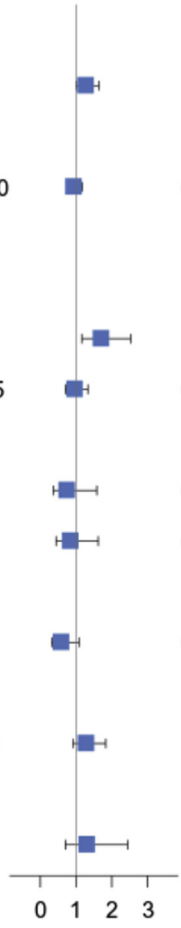
VEGFA145 is a secreted isoform that lacks exon 7 but retains a 24 aa fragment of exon 6.[12] This sequence mediates the binding of VEGFA145 to extracellular matrix and may allow for a role as a reservoir of angiogenic growth factors in the tumor stroma. VEGFA145 binds VEGFR2 and neuropilin receptor (NRP) 2 but not NRP1.[13] It acts as a mitogen but exhibits a reduced capacity to induce VEGFR2 phosphorylation compared to VEGFA165. This property may be related to the inability to recruit NRP1, which results in the shuttling of the VEGFR2-VEGFA145 complex from early endosomes toward late endosomes and lysosomal degradation.[14] Conversely, VEGFA145b plays a putative antiangiogenic role similar to other isoforms of the VEGFAxxxb family. The observed association of VEGFA145b to worse clinical outcome in patients receiving bevacizumab in our series hints at an inhibitory role in the tumor angiogenic microenvironment that is abrogated upon sequestration by bevacizumab. Alternatively, the excess of VEGFA145b may function by inactivating bevacizumab, thus preventing it from binding VEGFAxxxa and optimizing vasculature. Further basic research is warranted to elucidate its biological role.

Furthermore, we found that all VEGFAxxxb isoforms are negative prognosticators for right-sided primary tumors. In particular, high 121b is associated with worse PFS and OS, high 145b with worse PFS, and high 165b with worse OS in patients with right-sided colon cancer. This right-sided propensity is presumably related to differences in biology between right and left colon cancer. Right-sided and left-sided carcinomas exhibit distinct clinical and biological characteristics.[15,16] In particular, the distinct embryologic origin as well as the differences in microbiota and bile acid metabolites may play a significant role in cancer development. In addition, the mutational and gene expression profiles differ between right-sided and left-sided primary tumors. Microsatellite instability and *BRAF* mutations are more common in right-sided CRC, whereas *APC* and *TP53* mutations are enriched in left-sided primary tumors. Consequently, it is possible that right-sided colon cancer also bears a tumor angiogenic microenvironment that is regulated distinctly from left-sided colon angiogenesis and affects prognosis differently.

Alternative splicing and VEGFAxxxb formation is dependent on the tumor microenvironment and growth factor signaling. Preclinical evidence has shown that transforming growth factor beta (TGF-β) induced distal splice-site selection and up-regulation of VEGFAxxxb levels, whereas in vitro treatment with insulin-like growth factor, platelet-derived growth factor, and tumor necrosis factor alpha promoted proximal splice-site selection and reduction of VEGFAxxxb expression. p38-MAPK and Clk/sty are the responsible kinases that mediate the TGF-β1−induced distal site selection through modulation of SRp55 protein splice factor.[17] Interestingly, components of the TGF-β signaling are more frequently aberrated in right-sided than in left-sided colon cancer. Data from The Cancer Genome Atlas indicate a *TGF-βR2* mutation rate of 27.3% in the right side as opposed to 1.4% in the left side, and a *SMAD4* mutation rate of 15.2% compared to 9.7%.[15] The TGF-β signaling pathway can both suppress local tumor growth and promote epithelial−mesenchymal transition and distal spread.[18] Furthermore, in a human organoid culture model of CRC, the $BRAF^{V600E}$ mutation contributed to a mesenchymal phenotype upon stimulation with TGF-β.[19] Interestingly, *BRAF* mutation is more common in right-sided colon cancer (24.2% vs. 2.1%). In view of the role of TGF-β in VEGFA distal site selection, we speculate that high levels of VEGFAxxxb may be expressed in tumors with a hostile microenvironment where TGF-β activity is increased. Despite the increased levels of the antiangiogenic VEGF isoforms, the invasive potential of these tumors may not be counterbalanced. The role of VEGFA145b as an adverse prognosticator in right-sided primary tumors exclusively, and as a predictive factor for resistance to bevacizumab in right-sided primary tumors more robustly, may be related to the distinctive genetic makeup and TGF-β activity of this localization.

Figure 3 Multivariate Analysis Hazard Plot for PFS

Parameter	N patients	N events	HR (95% CI)	*p-value*
Tumor location				
right vs. left colon	99 vs. 259	87 vs. 241	1.28 (1.00, 1.64)	0.051
Gender				
female vs. male	140 vs. 218	128 vs. 200	0.94 (0.75, 1.17)	0.56
Treatment type*VEGFA 145b				0.049
VEGFA 145b at Bevacizumab treated				0.002
positive high vs. negative	53 vs. 60	52 vs. 55	1.71 (1.16, 2.53)	0.007
positive low vs. negative	171 vs. 60	152 vs. 55	0.97 (0.71, 1.33)	0.85
VEGFA 145b at non-Bevacizumab treated				0.75
positive high vs. negative	17 vs. 13	16 vs. 13	0.75 (0.36, 1.58)	0.45
positive low vs. negative	44 vs. 13	40 vs. 13	0.85 (0.44, 1.62)	0.61
Bevacizumab at positive high 145b				
No vs. Yes	17 vs. 53	16 vs. 52	0.59 (0.32, 1.08)	0.089
Bevacizumab at positive low 145b				
No vs. Yes	44 vs. 171	40 vs. 152	1.29 (0.91, 1.83)	0.16
Bevacizumab at negative 145b				
No vs. Yes	13 vs. 60	13 vs. 55	1.31 (0.70, 2.44)	0.40

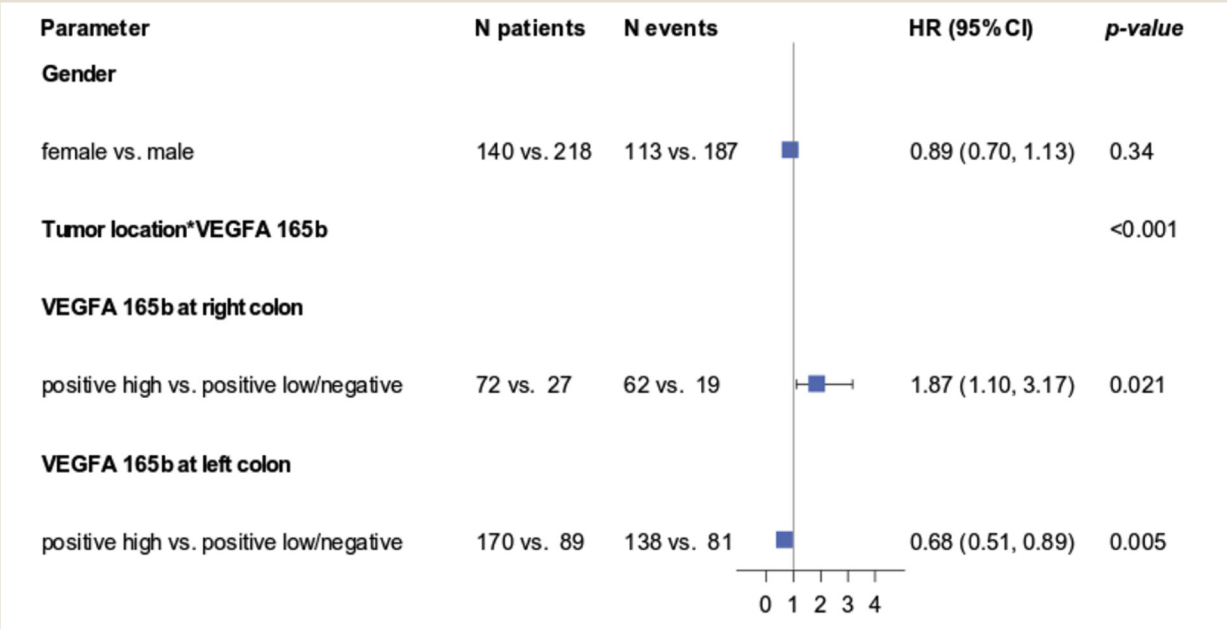


Abbreviation: PFS = progression-free survival.

In our data set, we showed that VEGFA165b was of favorable prognostic significance in left-sided primary tumors as opposed to right-sided primary tumors, in which it was associated with poorer prognosis. Data from an Italian prospective multicenter randomized phase 3 study indicated that only patients with right-sided colon cancer gained benefit from the addition of bevacizumab to chemotherapy.[20] In the same study, the authors found that patients with right-sided colon cancer had increased circulating levels of certain angiogenesis factors, whereas patients with left-sided colon cancer had more inflammatory indexes. VEGF165a is the prototypical VEGFA isoform that demonstrates the greatest affinity and efficacy on VEGFR2 by inducing maximal phosphorylation of the receptor, thereby stimulating endothelial cells and resulting in proliferation, migration, and sprouting.[14,21] However, VEGF165b shows similar affinity but much less efficacy on VEGFR2. In the proinvasive and redundant in angiogenic growth factor tumor environment of right-sided colon cancer, VEGFA165b may be contributing to a hypoxia-inducing prometastatic phenotype, whereas it may have a dominant antiangiogenic, anti-inflammatory, and antimetastatic role in left-sided tumors. Thus, VEGFA165b may be a double-edged sword depending on the biological context by inducing hypoxia, blocking bevacizumab action, or preventing angiogenic metastatic dissemination.[9]

Large VEGFA isoforms have the ability to bind the extracellular matrix, a property that allows for a slow and sustained release of angiogenic factors.[22] VEGFA tethering to extracellular matrix creates gradients of angiogenic factors modulated by proteolytic cleavage that result in a normalized vessel network, whereas freely diffusible VEGFA isoforms such as VEGFA121 lead to a chaotic vessel plexus.[5] VEGF145 and VEGF189 interact with heparin through residues encoded by exons 6a and 7, while VEGF165, which lacks exon 6a, binds heparin through exon 7–encoded residues. Furthermore, the differential interaction of the VEGF isoforms with NRP1 may play an additional role.[22] NRP1 is a coreceptor of VEGFR2 that mediates signaling by regulating VEGFR2 trafficking and expression. VEGF165a and VEGF189a bind NRP1; VEGF165b does not. The distinctive pharmacology of the VEGFA isoforms and the differing biology of left- and right-sided colon cancer may combine for the differential impact of the splice variants on prognosis.

Figure 4 Multivariate Analysis Hazard Plot for OS



Abbreviation: OS = overall survival.

The role of the VEGFA splice variants in prognosis has been evaluated in a limited number of studies. Bates et al[7] demonstrated that patients with metastatic colon cancer with low VEGFA 165b/total VEGFA ratio had better PFS when treated with a combination of chemotherapy with bevacizumab compared to chemotherapy alone. That was a retrospective study with patient samples derived from the E3200 trial, where patients were randomly assigned to receive FOLFOX (folinic acid, fluorouracil, and oxaliplatin) or FOLFOX with bevacizumab. Any discordance with our results may be related to different methodology, as in our study, we examined mRNA expression, whereas Bates et al assessed VEGFA expression by immunohistochemistry. Furthermore, in our study, patients received a combination of irinotecan and fluoropyrimidines, while patients in E3200 were treated with fluorouracil combined with oxaliplatin.

We have previously demonstrated that high VEGFAxxxa mRNA expression predicts a favorable outcome in breast cancer patients treated with a combination of bevacizumab and taxanes,[10] presumably because tumors with high VEGFAxxxa levels may be more angiogenic. Patients with increased VEGFAxxxb expression in their tumors had benefit from bevacizumab as well. These results are not directly comparable to ours here because in the breast cancer study, we examined the expression of larger VEGFA isoforms collectively as proangiogenic (xxxa) and antiantiogenic (xxxb), but we did not examine each isoform individually. We therefore could not draw a conclusion on the effects of VEGFA165, whether "a" or "b." Nevertheless, the present results support the proposed higher impact of the smaller xxxb in the antiangiogenic process.[23] Finally, disagreements with the findings of the CRC study could be attributed to differences in vascular biology between colon and breast cancer.[24]

Our study has several limitations. First of all, this investigation was not implemented within a randomized clinical trial, so it should be regarded as hypothesis generating pending confirmatory analysis. Moreover, despite the adequate sample size, the treatment groups were not even, as only 20.8% of patients were treated with chemotherapy alone. Systemic therapy was selected on the basis of the physician's choice, taking into consideration individual disease demographics and patient comorbidity/preferences, and so selection bias cannot be ruled out. Furthermore, the molecular study was performed in tumor samples obtained from the primary tumor in the majority of cases in order to study the impact of VEGFA splice variants in bevacizumab-based therapy of metachronous metastatic disease. Finally, the selection of cutoffs for classifying mRNA expression as high or low was based on value distribution characteristics within the study population; however, these are in need of independent prospective validation.

In conclusion, we report a negative predictive significance of VEGFA145b tumor mRNA expression for patients with mCRC treated with bevacizumab, which is more marked in right-sided primary tumors. In addition, all VEGFAxxxb isoforms were negative prognosticators for right-sided primary tumors, while VEGFA165b was of favorable prognostic significance for left-sided CRC. Because the lack of biomarkers for antiangiogenic therapies constitutes a serious impediment for the implementation of precision medicine, we believe that our data warrant further validation in independent prospective series.

Clinical Practice Points

- There is currently no available validated predictive biomarker of response to bevacizumab therapy in patients with mCRC.

- Although the target of bevacizumab is the VEGFA, the role of specific VEGFA isoforms and the mRNA expression of the respective splice variants have not been adequately examined.
- High VEGF145b expression predicted poor outcome in patients with mCRC—more markedly so in those with right-sided primary tumors.
- High expression of all VEGFAxxxb splice variants was associated with worse prognosis in patients with right-sided tumors, while high VEGF165b expression was related to favorable prognosis in patients with left-sided tumors.
- Our data warrant further validation in a well-powered independent population of patients with right- versus left-sided CRC tumors to examine the role of expression of splice variants in primary and metastatic sites.
- We speculate that validation of the aforementioned markers could protect patients from unnecessary treatment with bevacizumab or could permit selection of patients with poor prognosis requiring intensification with chemotherapy.

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Disclosure

G.P. discloses an advisory role for Roche; honoraria from Roche; speaker's bureau from Roche; and grants from Amgen. E.S. discloses an advisory board for Merck, MSD, Asta-Zeneca, Roche, Amgen, and Genesis. T.M. discloses an advisory role for Roche, Genesis Pharmaceuticals, and Novartis; honoraria from Roche, Merck, Gilead Sciences, Astra-Zeneca, and BMS; and travel from Merck, Pfizer, MSD, and Sanofi. P.P. discloses an advisory role for Roche, Merck, and Genesis Pharmaceuticals; and honoraria from Roche and Merck. D.B. discloses an advisory role for BMS, Roche, MSD, Pierre-Fabre, and Novartis. G.F. discloses advisory board for Pfizer, Sanofi, and Roche; and honoraria from Astra-Zeneca. The other authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental figure and tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2019.07.007.

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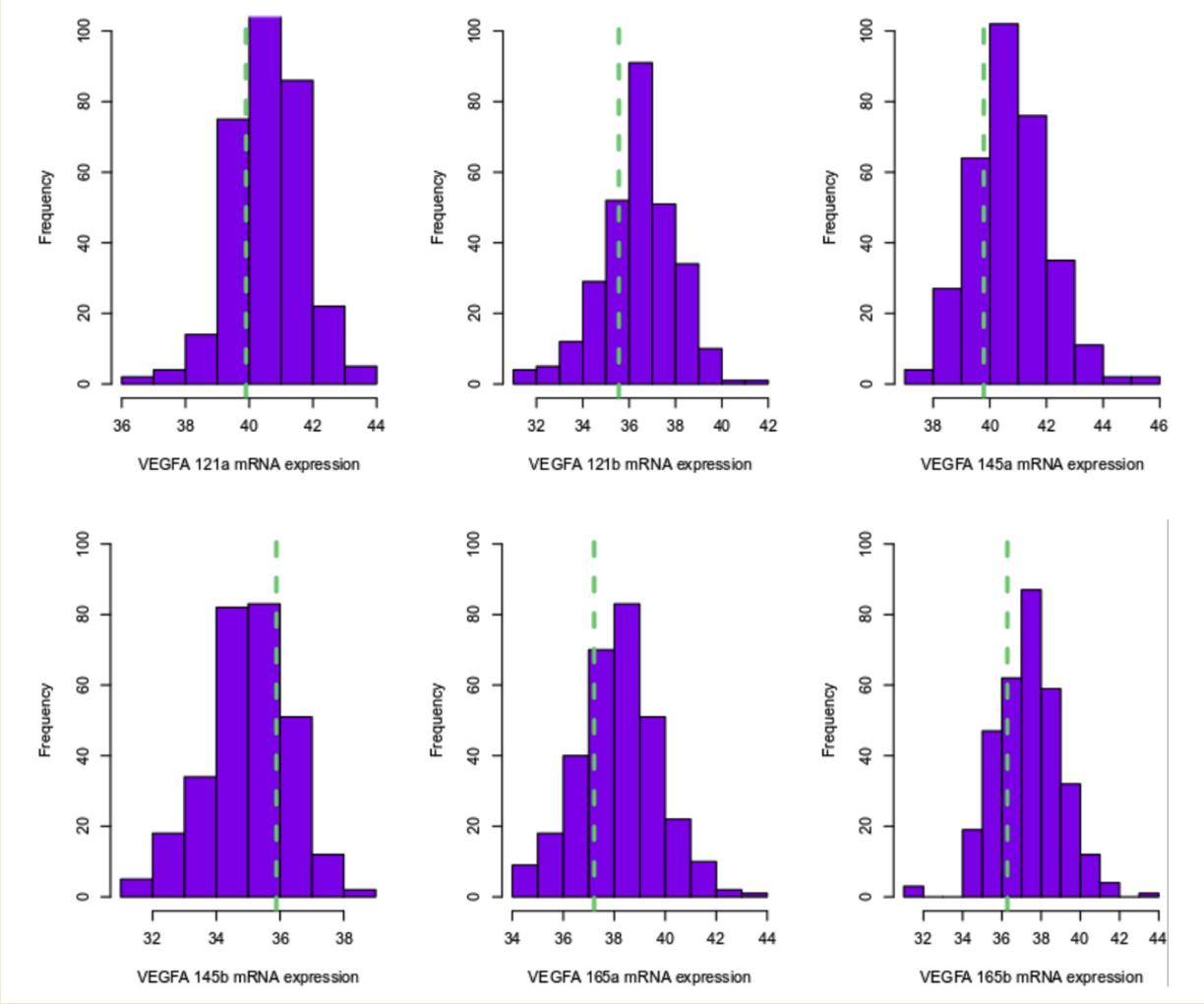
Supplemental Figure 1 Respective Distributions for All VEGFA Splice Variant Markers



Abbreviation: VEGFA = vascular endothelial growth factor A.

Supplemental Table 1 Associations of Markers' Expression With Clinicopathologic Parameters

		Gender			Age			Performance Status			Pathologic Primary Tumor (T)				Pathologic Regional Lymph Nodes (N)					Metastatic Site				No. of Metastatic Sites		
Parameter	Category	F	M	*P*	≤ 65 Years	> 65 Years	*P*	0	1-2	*P*	T1-T2	T3-T4	TX	*P*	N0	N1	N2	NX	*P*	Liver Only	Nodal	Visceral	*P*	1-2	≥ 3	*P*
VEGFA 121a mRNA expression	Negative	16 (11.3)	23 (10.5)	.900	24 (12.4)	15 (9.0)	.573	27 (10.8)	11 (10.8)	.502	5 (25.0)	29 (9.5)	2 (20.0)	.192	9 (11.3)	16 (13.0)	12 (9.2)	2 (11.8)	.510	21 (13.7)	3 (6.7)	8 (6.3)	.203	32 (10.1)	4 (14.3)	.273
	Positive high	92 (65.2)	148 (67.6)		126 (65.3)	114 (68.3)		163 (64.9)	72 (70.6)		10 (50.0)	208 (68.4)	6 (60.0)		56 (70.0)	73 (59.3)	91 (69.5)	13 (76.5)		99 (64.7)	34 (75.6)	86 (68.3)		217 (68.5)	15 (53.6)	
	Positive low	33 (23.4)	48 (21.9)		43 (22.3)	38 (22.8)		61 (24.3)	19 (18.6)		5 (25.0)	67 (22.0)	2 (20.0)		15 (18.8)	34 (27.6)	28 (21.4)	2 (11.8)		33 (21.6)	8 (17.8)	32 (25.4)		68 (21.5)	9 (32.1)	
VEGFA 121b mRNA expression	Negative	29 (20.6)	41 (18.7)	0.804	41 (21.2)	29 (17.4)	.582	50 (19.9)	20 (19.6)	.241	4 (20.0)	54 (17.8)	5 (50.0)	.094	17 (21.3)	18 (14.6)	27 (20.6)	6 (35.3)	.018	27 (17.6)	10 (22.2)	23 (18.3)	.803	66 (20.8)	3 (10.7)	.141
	Positive high	82 (58.2)	135 (61.6)		112 (58.0)	105 (62.9)		145 (57.8)	67 (65.7)		11 (55.0)	187 (61.5)	5 (50.0)		46 (57.5)	69 (56.1)	87 (66.4)	10 (58.8)		94 (61.4)	24 (53.3)	80 (63.5)		193 (60.9)	16 (57.1)	
	Positive low	30 (21.3)	43 (19.6)		40 (20.7)	33 (19.8)		56 (22.3)	15 (14.7)		5 (25.0)	63 (20.7)			17 (21.3)	36 (29.3)	17 (13.0)	1 (5.9)		32 (20.9)	11 (24.4)	23 (18.3)		58 (18.3)	9 (32.1)	
VEGFA 145a mRNA expression	Negative	16 (11.3)	21 (9.6)	0.813	20 (10.4)	17 (10.2)	.916	27 (10.8)	10 (9.8)	.546	3 (15.0)	31 (10.2)	2 (20.0)	.738	9 (11.3)	15 (12.2)	10 (7.6)	2 (11.8)	.524	19 (12.4)	4 (8.9)	10 (7.9)	.245	34 (10.7)	2 (7.1)	.799
	Positive high	95 (67.4)	147 (67.1)		128 (66.3)	114 (68.3)		165 (65.7)	73 (71.6)		13 (65.0)	204 (67.1)	7 (70.0)		51 (63.8)	76 (61.8)	97 (74.0)	12 (70.6)		93 (60.8)	31 (68.9)	93 (73.8)		210 (66.2)	20 (71.4)	
	Positive low	30 (21.3)	51 (23.3)		45 (23.3)	36 (21.6)		59 (23.5)	19 (18.6)		4 (20.0)	69 (22.7)	1 (10.0)		20 (25.0)	32 (26.0)	24 (18.3)	3 (17.6)		41 (26.8)	10 (22.2)	23 (18.3)		73 (23.0)	6 (21.4)	
VEGFA145b mRNA expression	Negative	22 (15.6)	51 (23.3)	0.062	42 (21.8)	31 (18.6)	.457	54 (21.5)	19 (18.6)	.831	3 (15.0)	62 (20.4)	3 (30.0)	.811	12 (15.0)	31 (25.2)	24 (18.3)	3 (17.6)	.413	35 (22.9)	7 (15.6)	22 (17.5)	.007	66 (20.8)	3 (10.7)	.143
	Positive high	35 (24.8)	36 (16.4)		41 (21.2)	30 (18.0)		47 (18.7)	20 (19.6)		5 (25.0)	56 (18.4)	1 (10.0)		16 (20.0)	24 (19.5)	25 (19.1)	1 (5.9)		20 (13.1)	6 (13.3)	37 (29.4)		62 (19.6)	3 (10.7)	
	Positive low	84 (59.6)	132 (60.3)		110 (57.0)	106 (63.5)		150 (59.8)	63 (61.8)		12 (60.0)	186 (61.2)	6 (60.0)		52 (65.0)	68 (55.3)	82 (62.6)	13 (76.5)		98 (64.1)	32 (71.1)	67 (53.2)		189 (59.6)	22 (78.6)	
VEGFA 165a mRNA expression	Negative	25 (17.7)	29 (13.2)	0.381	30 (15.5)	24 (14.4)	.396	39 (15.5)	14 (13.7)	.093	6 (30.0)	44 (14.5)	1 (10.0)	.031	16 (20.0)	15 (12.2)	20 (15.3)	2 (11.8)	.094	22 (14.4)	4 (8.9)	22 (17.5)	.622	48 (15.1)	4 (14.3)	.629
	Positive high	84 (59.6)	145 (66.2)		127 (65.8)	102 (61.1)		151 (60.2)	73 (71.6)		8 (40.0)	197 (64.8)	4 (40.0)		47 (58.8)	75 (61.0)	91 (69.5)	8 (47.1)		96 (62.7)	31 (68.9)	81 (64.3)		203 (64.0)	16 (57.1)	
	Positive low	32 (22.7)	45 (20.5)		36 (18.7)	41 (24.6)		61 (24.3)	15 (14.7)		6 (30.0)	63 (20.7)	5 (50.0)		17 (21.3)	33 (26.8)	20 (15.3)	7 (41.2)		35 (22.9)	10 (22.2)	23 (18.3)		66 (20.8)	8 (28.6)	
VEGFA 165b mRNA expression	Negative	15 (10.6)	19 (8.7)	0.747	18 (9.3)	16 (9.6)	.739	24 (9.6)	9 (8.8)	.099	4 (20.0)	28 (9.2)	1 (10.0)	.244	12 (15.0)	11 (8.9)	9 (6.9)	2 (11.8)	.010	17 (11.1)	2 (4.4)	10 (7.9)	.634	30 (9.5)	3 (10.7)	.688
	Positive high	96 (68.1)	148 (67.6)		134 (69.4)	110 (65.9)		162 (64.5)	77 (75.5)		9 (45.0)	209 (68.8)	6 (60.0)		44 (55.0)	77 (62.6)	104 (79.4)	11 (64.7)		104 (68.0)	33 (73.3)	85 (67.5)		217 (68.5)	17 (60.7)	
	Positive low	30 (21.3)	52 (23.7)		41 (21.2)	41 (24.6)		65 (25.9)	16 (15.7)		7 (35.0)	67 (22.0)	3 (30.0)		24 (30.0)	35 (28.5)	18 (13.7)	4 (23.5)		32 (20.9)	10 (22.2)	31 (24.6)		70 (22.1)	8 (28.6)	

Data are presented as n (%).
Abbreviations: mRNA = messenger RNA; VEGFA = vascular endothelial growth factor A.

Supplemental Table 2 Patient Outcome by Treatment Type and Tumor Location

Characteristic	Treatment		Tumor Location	
	Irinotecan and Bevacizumab (N = 285)	Irinotecan Only (N = 75)	Left Colon (N = 259)	Right Colon (N = 99)
Follow-up (mos)	99.4 (89.31-107.93)	133.5 (93.48-140.82)	106.1 (96.98-123.44)	91.6 (46.79-115.70)
PFS (mos)	10.8 (9.87-11.41)	10.1 (8.95-12.23)	11.3 (10.36-12.39)	9.2 (7.44-10.10)
Progression	214 (75.09)	62 (82.67)	202 (77.99)	73 (73.74)
Survival (mos)	26.1 (23.44-29.51)	25.0 (16.95-31.93)	29.2 (25.61-32.30)	18.2 (16.39-24.30)
Death	235 (82.46)	66 (88.00)	219 (84.56)	81 (81.82)

Data are presented as median (95% confidence interval) and n (%).