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Prognostic Value of Vascular Endothelial Growth Factor Tumor Tissue Content of Colorectal Cancer

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Key Words
Vascular endothelial growth factor · Tumor tissue · Longitudinal study · Prognosis · Survival analysis

Abstract
Objectives: A longitudinal study was designed to quantify tumor tissue content of vascular endothelial growth factor (VEGF) in patients with colorectal cancer (CRC) and to evaluate its prognostic value in respect to the relapse-free and overall survivals. ***Methods:*** Sixty-nine patients with CRC were followed from the time of diagnosis of primary tumor for at least 3 years after surgery. Quantitative evaluation of VEGF content in tissue was performed on whole protein extracts obtained from biopsies of histologically confirmed neoplastic tissues and corresponding mucosa, histologically confirmed as 'normal'. ***Results:*** VEGF levels were higher in CRC tissues, median 141 pg/mg of protein (interquartile range 70–375), compared with corresponding normal mucosa, median 45 pg/mg of protein (interquartile range 22–78; $p < 0.0001$), and were associated with the stage of disease ($p = 0.035$) by multivariate analysis. Tumor VEGF content was higher in relapsing patients compared with those who remained disease free ($p < 0.001$) and was independently associated with relapse-free survival ($p = 0.044$). Cox's proportional hazard survival analysis demonstrated that VEGF ($p = 0.035$) had an independent prognostic value in respect to overall survival. ***Conclusions:*** Elevated tumor VEGF content may discriminate between early and late stages of CRC and may be used as an independent prognostic parameter in the management of these patients.

Introduction

Colorectal cancer (CRC) constitutes a public health burden worldwide. Currently, the most important factor predictive of survival is the regional lymph node status at the time of initial surgery. However, this is not sufficient to accurately predict outcomes, since approximately 20% of stage B patients (i.e. without regional lymph node involvement) will not be alive 5 years after curative resection. Thus, it would be of clinical relevance to identify novel markers of prognosis that may help improve the clinical outcome of CRC.

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In this respect, vascular endothelial growth factor (VEGF) has received much attention in the past few years. VEGF is the predominant angiogenic factor in human CRC, and its level of expression (assessed by a variety of techniques including Northern blotting, immunohistochemistry, enzyme-linked immunosorbent assay and/or RT-PCR) has been shown to correlate with tumor development and progression, metastasis and/or tumor vascularity [1–5]. Moreover, by immunohistochemical analysis, it has been demonstrated that VEGF is upregulated in progression from nonmalignant to malignant colon cancers [6, 7] and that the angiogenic switch may occur between Tis and T1, i.e. simultaneous with the initiation of invasion [8]. Thus, it appears that VEGF may be activated in the early stages of colorectal tumor development and is important in both tumor progression and metastases. Nevertheless, analyses relating VEGF to survival for prognostic use are conflicting [9–11], also depending on the technique used to quantitate VEGF expression.

To date, immunohistochemistry is considered sufficiently reliable to provide meaningful information for patients' prognosis in clinical trials. However, this approach cannot be generally used for clinical decision making, mainly because it is limited by intra- and interobserver variations [12], and categorizing continuous variables risks discarding potentially meaningful information [13]. Quantitation imposes a greater degree of objectivity to the measurement, is more reproducible than perceptually difficult subjective discriminations and facilitates the communication and clarity of definitions [12].

The ability to quantitate the actual antigen content within tumor tissue provides a new perspective on the biology of tumor-associated antigens [14, 15], and some newly proposed and investigated molecular markers appear promising [3], but not all laboratories are equipped for their determination. For this reason, we thought it would have been of interest to investigate the actual VEGF content on protein extracts from tumor and corresponding normal mucosa tissues obtained from patients with primary CRC at the time of surgery. To this purpose, a longitudinal study was designed to quantify VEGF tumor tissue content in patients with different stages of CRC and to evaluate its possible correlation with clinicopathological features in respect to prognostic information on the relapse-free and overall survivals.

Patients and Methods

Patients and Sample Collection

Sixty-nine consecutive patients with histologically diagnosed primary colorectal adenocarcinoma, treated at the Department of Surgery of the University of Rome Tor Vergata, entered into the study. None of the patients received neoadjuvant chemotherapy or radiation therapy before surgery, and none received antiangiogenic agents at any time of the study. Tumor resection was carried out in all patients, and simultaneous partial hepatectomy for single liver metastasis was performed in 5 patients. Fifty (72%) of 69 patients were considered eligible for adjuvant therapy. In particular, 2 of 12 patients with stage B1 CRC accepted to be included in the adjuvant therapy protocol, whereas 3 of 25 stage B2 patients were not considered eligible due to advanced age or comorbidity. Adjuvant therapy was instituted in all stage C patients (n = 21) and first-line chemotherapy was instituted in the 5 stage D patients. Demographic and clinical information are reported in table 1. Patients were followed from the time of diagnosis of primary tumor for at least 3 years after surgery or until time of recurrence. All patients were generally reviewed at 3-month intervals during the first 2 years after surgery. Thereafter, the interval between visits increased to 6 or 12 months in parallel with tumor stage. No patient was lost at follow-up. The study was performed under the appropriate institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki. Informed consent was obtained from each participating subject.

Multiple biopsies of histologically confirmed neoplastic tissues and mucosa (peeled from colonic wall approximately 10 cm distant to the tumor), histologically confirmed as 'normal', were obtained at surgery, immediately frozen in liquid nitrogen and subsequently evaluated for VEGF content. All carcinoma specimens were shown to be free of necrotic areas and to contain at least 50% malignant cells by histopathology. Furthermore, all specimens designated as 'normal mucosa' from carcinoma patients contained at least 80% colonic mucosa. The quantitative evaluation of VEGF content in tissue was performed on whole protein extracts obtained from all the biopsies.

Preparation of Tissue Protein Extracts and VEGF Determination

The procedure for protein extraction has been described in detail elsewhere [14, 15]. Briefly, all human colorectal tissues were homogenized with an OMNI 1000 homogenizer (OMNI, Waterbury, Conn., USA) at high speed (20,000 rpm). The tissues were initially resuspended at a ratio of 1:10 g tissue/ml in buffer containing 10 m*M* Tris-HCl, pH 7.2, 0.2 m*M* $CaCl_2$ (extraction buffer). The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was removed and sonicated at 4°C for 1 min at 15-second intervals. The sonicate was centrifuged at 10,000 *g* for 10 min, and the protein concentration of the supernatant was determined by the method of Lowry.

The VEGF tissue content was measured by a commercially available enzyme immunoassay (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. The assay has been reported to recognize both natural and recombinant human VEGF and not to exhibit cross-reactivity with a series of cytokines and growth factors. Intra- and interassay coefficients of variation are below 10%. The minimum detectable dose is lower than 9.0 pg/ml. In our experimental conditions, the linear correla-

Table 1. Clinical features of 69 CRC patients at time of surgery for primary tumor: comparison between patients who relapsed and patients who remained free of disease (NED) during postsurgical follow-up

Variable	Whole series n = 69	NED n = 51	p value	Relapsing n = 18
Age, years				
Mean ± SD	62 ± 11	61 ± 10	NS	63 ± 11
Range	35–80	35–80		37–80
Males	39 (57)	32 (63)	NS	7 (39)
Site of primary tumor			NS	
Colon	26 (38)	19 (37)		7 (39)
Sigma	19 (27)	15 (30)		4 (22)
Rectum	24 (35)	17 (33)		7 (39)
Dukes' stage			<0.001	
A	6 (9)	6 (12)		0 (0)
B	37 (54)	33 (65)		4 (22)
C	21 (30)	10 (19)		11 (61)
D[1]	5 (7)	2 (4)		3 (17)
Total	69	51		18
Adjuvant therapy	50 (72)	33 (65)	<0.02	17 (94)
Follow-up, months				
Median		76.3	<0.001	37.7
Range		40.5–153.1		14.6–99.6
Mode of recurrence				
Locoregional				3 (16.7)
Peritoneum				7 (38.9)
Liver				7 (38.9)
Lung				1 (5.6)

Figures in parentheses are percentages.
[1] Patients with a synchronous liver metastasis.

tion coefficient between all duplicates tested by a single operator in a single assay was always >0.990. All protein extracts obtained from colorectal tissues were initially diluted with extraction buffer to a protein concentration of 1.0 mg/ml. The concentrations of VEGF in the tumor tissues and normal mucosas are expressed in picogram per milligram protein. Measurements were done blinded. Results were calculated from a standard curve using recombinant human VEGF in the range of 31.2–2,000 pg/ml. All samples were assayed in duplicate and those showing values above the standard curve were serially diluted in protein extraction buffer, and the test was repeated. The value for each sample was calculated as the mean of at least two determinations of three different biopsies from the same sample.

Statistical Analysis

Unless otherwise specified, data are presented as median and interquartile ranges (IQR). Differences between groups were assessed by Wilcoxon's matched pairs test, the Mann-Whitney U test and Kruskal-Wallis test. Univariate and multivariate analyses were performed by Cox's proportional hazard model: the first step was performed by the log-rank test, then the covariates found to be associated with recurrences were included in the Cox regression model. For each variable, the proportional hazard has been tested. Clinical and laboratory variables considered in the analysis were: sex, site of primary tumor, grading, Dukes' stage, tumor tissue and normal mucosa VEGF content. The latter two variables were categorized according to a cut-off of 215 pg/mg of protein (i.e. the 90th percentile of the antigen content of colorectal normal mucosa, yielding a specificity greater than 90%). The variables that achieved statistical significance in the univariate analysis were subsequently included in a multivariate analysis using a Cox regression model. Only p values lower than 0.05 were regarded as statistically significant. All calculations were made using computer software packages (EGRET Cytel Software Co., Cambridge, Mass., and Statistica, StatSoft Inc., Tulsa, Okla., USA).

Results

Tissue VEGF content was determined on protein extracts obtained from CRC and corresponding normal mucosa biopsies sampled at the time of surgery from all 69 patients recruited. As shown in figure 1, median VEGF levels were higher in CRC tissues (141 pg/mg of protein, IQR 70–375) compared with the corresponding normal mucosa (45 pg/mg of protein, IQR 22–78; $p < 0.0001$). Moreover, the tissue VEGF content was above the cut-off

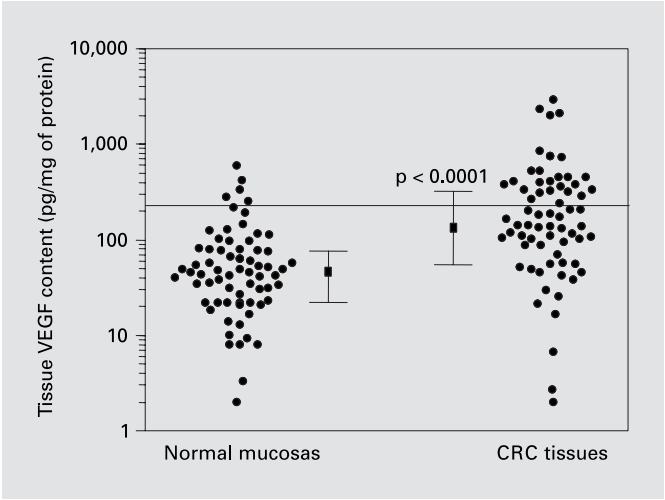

Fig. 1. Scatterplot distribution of tissue VEGF contents in tumor and corresponding normal mucosa biopsies of 69 CRC patients. Black bars represent median values; whiskers indicate interquartile ranges. The dotted line represents the 90th percentile of the values observed in normal mucosa specimens.

Table 2. Association between clinicopathological variables and tumor tissue and corresponding normal mucosa VEGF content in 69 CRC patients

Variable	Patients	Positive levels of VEGF[1]			
		Tumor tissue	p value	Normal mucosa	p value
Site of primary tumor					
Colon	26	11 (42.3)		3 (11.5)	
Rectum	24	9 (37.5)		0 (0.0)	
Sigma	19	7 (36.8)	0.92	2 (10.5)	0.24
Grading					
1	18	2 (11.1)		0 (0.0)	
2	42	19 (45.2)		5 (11.9)	
3	9	6 (66.7)	0.009	0 (0.0)	0.18
Tumor size					
T1–2	21	3 (14.3)		1 (4.8)	
T3–4	48	24 (50.0)	0.005	4 (8.3)	0.60
Lymph node involvement					
N0	43	12 (27.9)		3 (7.0)	
N+	26	15 (57.7)	0.014	2 (7.7)	0.91
Dukes' stage					
A	6	1 (16.8)		1 (16.7)	
B	37	11 (29.7)		2 (5.4)	
C	21	11 (52.4)		2 (9.5)	
D	5	4 (80.0)	0.053	0 (0.0)	0.68

Figures in parentheses are percentages.
[1] Above the cut-off value of 215 pg/mg of protein.

value of 215 pg/mg of protein in 39% (27 of 69) of CRC specimens compared with approximately 7% (5 of 69) of the corresponding normal mucosa specimens. No association was found between normal mucosa VEGF content and clinicopathological variables. Notably, of the 5 patients with elevated normal mucosa VEGF content, 3 had a family history of cancer and 1 developed a second primary tumor of the transverse colon approximately 10 years after resection of a stage A CRC of the ascending colon. The last patient (C2) had liver metastasis 1.5 years after initial diagnosis and died of disease.

The tumor tissue VEGF content was significantly associated with grading, tumor size, lymph node involvement and the stage of disease (table 2). Thus, to further analyze the relationship between tumor tissue VEGF content and clinicopathological variables of CRC, a multiple regression analysis including age, sex, grading, tumor size, lymph node involvement, stage and normal mucosa VEGF content was carried out. The final model obtained by stepwise regression analysis revealed that stage of disease (regression coefficient = 0.25; $p = 0.035$) and normal mucosa VEGF content (regression coefficient = 0.23; $p = 0.045$) were independently related to VEGF content of CRC tissues (R^2 for the entire model = 0.18; $p = 0.015$). Figure 2 reports the distribution of tissue VEGF content in CRC specimens obtained from the primary tumor at the time of surgery in comparison with the values measured in the corresponding normal mucosa and in tumor biopsies sampled at the metastatic site in the 5 patients with synchronous liver metastasis. As shown, the VEGF content of normal mucosas was significantly lower than that measured in CRC tissues obtained from stage A patients. Furthermore, the tumor VEGF content at the metastatic site was significantly higher than that measured in the corresponding primary tumor (fig. 2).

Patients were longitudinally monitored during postsurgical follow-up for either a minimum of 3 years or until time of recurrence. Fifty-one (74%) of the 69 patients remained free of disease throughout follow-up, while 18 patients experienced recurrence of disease (table 1). No differences were observed in age, sex and histotype or site of primary tumor between patients with and without recurrence (table 1). The median tumor tissue VEGF content was higher in relapsing patients compared with those who remained free of disease throughout the follow-up (median 427 pg/mg of protein, IQR 137–737, vs. 130 pg/mg of protein, IQR 48–308; $p < 0.001$). Moreover, tumor tissue VEGF content was elevated in ap-

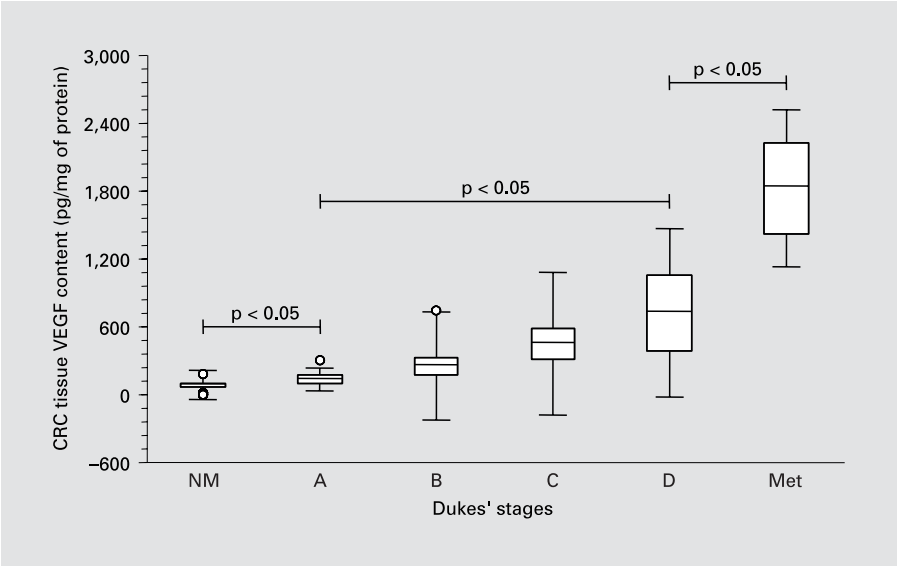

Fig. 2. Box plot analysis of tumor tissue VEGF content in 69 CRC patients stratified on the basis of the stage of disease. Comparison with VEGF content of normal mucosa (NM) and tumor biopsies obtained from synchronous metastasis (Met). Data are presented as mean values (solid lines), standard deviations (columns) and standard errors of the mean (whiskers). Open circles indicate outliers.

proximately 72% of relapsing patients compared with 28% ($p < 0.001$) of patients who remained free of disease (table 3). Univariate analysis of clinicopathological variables and tumor tissue content of VEGF demonstrated that relapse-free survival was affected by Dukes' stage of disease ($p < 0.001$), grading ($p = 0.039$) and tumor tissue VEGF content ($p = 0.044$) (table 4). However, multivariate analysis obtained by the Cox regression model confirmed that only Dukes' stage had an independent prognostic role in predicting relapsing disease ($p < 0.001$) (table 4). Given that chemotherapy is known to influence the eventual relapse of colon cancer and that 72% of our patients were included in such protocols, we repeated the analysis after applying to the same model a risk set stratification for the variable 'chemotherapy'. The result obtained confirmed that relapse-free survival was affected by tumor tissue VEGF content (log-rank $\chi^2 = 3.9$; $p = 0.05$) at the univariate analysis independently of treatment.

To further analyze the prognostic value of tumor tissue VEGF content in CRC, Cox's proportional hazard survival analysis of the overall survival was also performed. As shown in table 5, Dukes' stage ($p = 0.007$) and VEGF ($p = 0.035$) had an independent prognostic value, which was confirmed to be independent of chemotherapy administration (hazard ratio for VEGF tumor tissue content = 5.15; 95% CI 1.1–24.1; $p = 0.038$). Moreover, when risk set stratification for Dukes' stage of disease was applied to the model, only tumor tissue VEGF content (hazard ratio = 4.8; 95% CI 1.1–22.1; $p = 0.044$) had an independent prognostic role in respect to overall survival. Figure 3 demonstrates the Kaplan-Meier curves for patients with positive (above the cut-off value) or negative (below the cut-off value) VEGF tumor tissue content. As shown, positive VEGF levels were associated with an increased incidence of recurrence (log-rank statistic = 8.27; $p = 0.004$) (fig. 3a) and a higher mortality rate (log-rank statistic = 8.56; $p = 0.003$) (fig. 3b) compared with patients with negative levels of this variable.

Table 3. VEGF tumor tissue and normal mucosa content in 69 primary CRC patients: comparison between relapsing patients and patients who remained free of disease during postsurgical follow-up

VEGF	NED n = 51	Relapsing n = 18	p value
Tumor tissue			
<215 pg/mg protein	37 (72.6)	5 (27.8)	
>215 pg/mg protein	14 (27.5)	13 (72.2)	0.0001
Normal mucosa			
<215 pg/mg protein	48 (94.1)	16 (88.9)	
>215 pg/mg protein	3 (5.9)	2 (11.1)	0.16

Figures in parentheses are percentages.

Table 4. Cox's proportional hazard analysis of relapse-free survival in 69 primary colorectal cancer patients

Factors		Patients	Recurrences	Univariate		Multivariate	
				log-rank χ^2	p value	hazard ratio (95% CI)	p value
Sex	Females	30	11 (37)				
	Males	39	7 (18)	3.397	0.065		
Site	Colon	26	7 (27)				
	Sigma	19	4 (21)				
	Rectum	24	7 (29)	0.366	0.545		
Dukes' stage	A	6	0 (0)				
	B	37	4 (11)				
	C	21	11 (52)				
	D	5	3 (60)	19.48	<0.001	3.45 (1.94–6.17)	<0.001
Grading	1	18	1 (7)				
	2	42	14 (33)				
	3	9	3 (33)	4.266	0.039		
Tumor VEGF	<215 pg/mg	43	6 (14)				
	>215 pg/mg	26	12 (46)	4.046	0.044		
Normal mucosa VEGF	<215 pg/mg	63	15 (24)				
	>215 pg/mg	6	3 (50)	0.491	0.484		

Figures in parentheses are percentages.

Table 5. Cox's proportional hazard analysis of mortality rates in 69 primary colorectal cancer patients

Factors		Patients	Deaths	Univariate		Multivariate	
				log-rank χ^2	p value	hazard ratio (95% CI)	p value
Sex	Females	30	9 (39)				
	Males	39	5 (13)	3.377	0.066		
Site	Colon	26	6 (23)				
	Sigma	19	3 (16)				
	Rectum	24	5 (21)	0.383	0.536		
Dukes' stage	A	6	0 (0)				
	B	37	4 (11)				
	C	21	8 (38)				
	D	5	2 (40)	8.556	0.003	2.72 (1.32–5.62)	0.007
Grading	1	18	0 (0)				
	2	42	11 (26)				
	3	9	3 (33)	6.707	0.010		
Tumor VEGF	<215 pg/mg	43	4 (9)				
	>215 pg/mg	26	10 (39)	4.801	0.028	5.27 (1.12–24.8)	0.035
Normal mucosa VEGF	<215 pg/mg	63	11 (18)				
	>215 pg/mg	6	3 (50)	0.975	0.323		

Figures in parentheses are percentages.

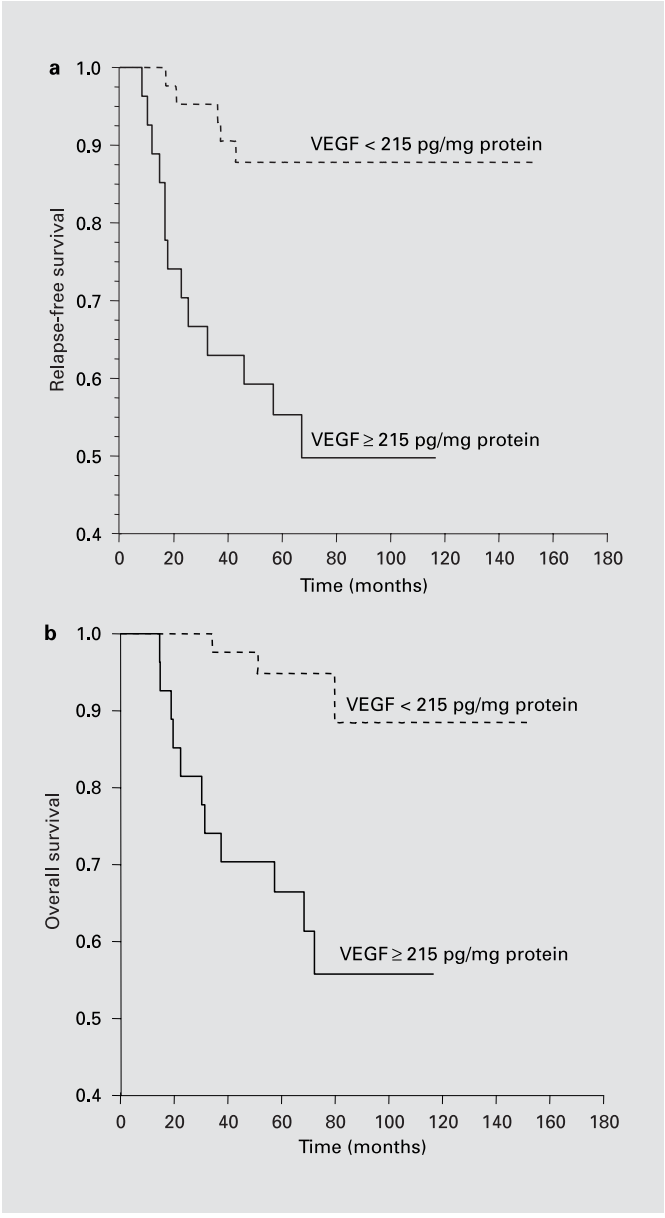

Fig. 3. Kaplan-Meier analysis of relapse-free (**a**) (log-rank statistic = 8.27; p = 0.004) and overall (**b**) (log-rank statistic = 8.56; p = 0.003) survival time. Comparison between patients with (solid line) and without (dotted line) positive VEGF (above 215 pg/mg of protein) tumor tissue content.

Discussion

To our knowledge, this is the first study in which the predictive impact of the actual VEGF content within tumor tissue has been analyzed in primary CRC. We demonstrated that in patients undergoing radical surgery for CRC, elevated tumor VEGF concentrations predicted a reduced disease-free and overall survival.

To date, VEGF is considered an adverse predictive factor in human cancer; however, the prognostic value of VEGF in CRC is still debated. Indeed, some studies suggested that tumor VEGF expression might be considered an independent prognostic factor for both disease-free [9, 16–18] and overall survival [18, 19] in patients with CRC, whereas other authors obtained negative results [10, 11]. This discrepancy might be largely dependent on the scoring method and categorization of variables used to review immunohistochemical analyses, which risk discarding potentially meaningful information. In the present study, tumor tissue content of VEGF was quantified on protein extracts obtained from colorectal tumor and corresponding normal mucosa biopsies, using the same immunoassay developed for serum level determination.

There is only one other study using a similar approach to analyze the association of tumor VEGF content with various factors reflecting the general condition of the patients, such as nutritional status or systemic oxygenation [20]. In agreement with their findings, the present study demonstrated that corresponding normal mucosa of CRC patients had a significantly lower VEGF content than tumor tissues. Nonetheless, approximately 7% of normal mucosa specimens had an elevated VEGF content. A familiar history of cancer was recorded in 3 of 5 patients, whereas 1 patient developed a second primary CRC. These findings are in agreement with the demonstration that VEGF is upregulated in progression from nonmalignant to malignant colon cancers [6, 7], and we might hypothesize that molecular changes involving VEGF expression may predispose a particular individual to develop neoplastic disease during his/her lifetime. Specific studies are currently needed to address this particular issue.

VEGF concentrations measured in protein extracts obtained from normal mucosa specimens were not associated with clinicopathological variables. Conversely, tumor VEGF content was associated with tumor size, grading, lymph node involvement and Dukes' stages of disease.

Taken together, these results indicated that the presence of an elevated tumor VEGF content may identify within different tumor stages a subset of CRC patients at high risk and may have a prognostic role in the management of these patients. This concept was further supported by the findings obtained in the postsurgical follow-up. In fact, 72% of the patients who developed recurrent disease had an elevated tumor VEGF content, compared

with approximately 28% of patients who remained free of disease. Furthermore, median tumor VEGF content was significantly higher in relapsing patients compared with patients who remained free of disease throughout the follow-up. Additionally, in univariate survival analyses, an elevated tumor tissue content of VEGF was associated with a worse relapse-free and overall survival. Moreover, in the multivariate analysis, positive VEGF values independently predicted a reduced overall survival, thus suggesting that the determination of the actual tumor tissue VEGF content might have a prognostic value in patients with CRC.

Our findings are also consistent with studies performed on serum VEGF levels, indicating that this cytokine may be useful for evaluating disease status and prognosis in patients with CRC [21–23]. However, there has been a debate about whether serum VEGF levels actually reflect tumor expression of VEGF [24–27]. Megakaryocytes are known to express VEGF, which is stored in the granules of the platelets and released during platelet activation [28]. Comparison of serum and plasma VEGF levels in the same cancer patient has shown much higher serum levels [24, 29], suggesting that most VEGF in the serum is derived from the platelets during clotting [30]. For all these reasons, no consensus has been reached so far on which specimen is to be used for an accurate assessment of circulating VEGF, although serum VEGF determination is still the most widely used. In our opinion, the quantification of the actual VEGF content on protein extracts from tumor tissues may be of advantage over the determination of circulating VEGF levels and might help in the choice of more aggressive treatment and/or more strict follow-up procedures in a subgroup of high-risk patients. Furthermore, the reported finding of an adverse prognostic value of VEGF in patients with CRC may have important implications if VEGF is used as a target in specific antiangiogenic therapy protocols, including VEGF antisense, monoclonal antibodies and specific small molecule inhibitors. For all of these approaches, our results indicate that tumor tissue VEGF content determination might also be employed as a criterion for patient entry in newly designed clinical trials to provide definitive evidence for their antitumor activity in humans.

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