Cancer Growth and Metastasis

ORIGINAL RESEARCH

Preoperative Vascular Endothelial Growth Factor Levels as a Prognostic Marker for Stage II or III Colorectal Cancer Patients

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Abstract

Background: The aim of the present study was to determine whether serum vascular endothelial growth factor (VEGF) can provide prognostic information independent of carcinoembryonic antigen levels in patients undergoing curative surgery.

Methods: Serum samples were collected from 158 patients with colorectal cancer and from 100 controls. Serum and tissue levels of VEGF were measured by enzyme-linked immunosorbent assay. Serum VEGF levels in colorectal cancer patients were compared with those in healthy controls, and we retrospectively assessed the association between serum VEGF levels and clinicopathologic findings and survival.

Results: VEGF expression was significantly higher in colorectal cancer tissue compared with nontumor tissue. Mean serum VEGF levels in patients were significantly higher than those in controls, and significantly higher in patients with large tumors, lymph node involvement, and distant metastases.

Conclusion: Elevated serum VEGF was significantly associated with poor survival, but was only an independent risk factor for poor survival in Stage II and/or III disease. Elevated serum VEGF is significantly associated with development of colorectal cancer, and lymph or distant invasive phenotypes and survival, especially in Stage II and III patients.

Keywords: colorectal cancer, vascular endothelial growth factor, carcinoembryonic antigen, prognostic factors, Stage II, Stage III

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Introduction

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide.[1] After potential curative surgery, approximately 30% of patients eventually develop metastases, often in spite of adjuvant therapies, including chemotherapy and radiochemotherapy.[2]

The main factors that determine the prognosis in colorectal cancer are lymph node involvement, tumor size, and local dissemination of disease.[3] However, these factors do not fully predict individual clinical outcomes, especially in patients with Stage II or Stage III disease.[1] Although adjuvant chemotherapy confers a significant survival benefit in Stage III patients, it is controversial whether this treatment has any effect in patients with Stage II colorectal cancer, 20%–30% of whom eventually experience tumor relapse.[4] Adjuvant chemotherapy has been shown to increase survival in certain populations of Stage II patients.[5] Furthermore, almost 60% of Stage III patients do not relapse, even if adjuvant chemotherapy is not given.[6] Therefore, identification of high-risk Stage II and III colorectal cancer patients would be of great benefit in selecting appropriate candidates for standard or intense adjuvant therapy.

Carcinoembryonic antigen (CEA) is a complex glycoprotein that is upregulated in approximately 90% of advanced colorectal cancers and contributes to the malignant characteristics of these tumors.[7] However, it is not useful for detecting asymptomatic cancer, because the sensitivity of CEA for early colorectal cancer is as low as 30%–40%.[8] Moreover, CEA is not significantly associated with survival in patients with Stage I or II lesions, and CEA testing is relatively insensitive to tumors with local or peritoneal involvement.[9]

Many angiogenic factors have been identified,[9] but so far the role and contribution of each of these in tumoral angiogenesis is not clear. Vascular endothelial growth factor (VEGF) seems to be one of the most important mediators of both normal and tumoral angiogenesis,[10] and the good preclinical results obtained by blocking VEGF and its receptors indicate that this factor may have a leading position in the hierarchy of mediators of angiogenesis.[9,10]

The growth of most solid tumors and their subsequent metastases depends critically on angiogenesis, ie, formation of new blood vessels in the endothelium of existing blood vessels. This process is tightly controlled by both stimulatory and inhibitory factors.[10] Vascular endothelial growth factor-A (VEGF-A) is one of the most potent angiogenic cytokines known, and is the pre-eminent member of a large family of factors. Several polymorphisms have been described in the VEGF-A gene that can potentially alter VEGF production and/or activity, causing differences in lymphangiogenesis and lymphatic metastasis.[11–13] However, with regard to lymphangiogenesis, it is VEGF-C rather than VEGF-A which appears to be the critical stimulatory factor.[10]

VEGF-A is a homodimeric heparin-binding glycoprotein with a molecular mass of 34–42 kDa. It stimulates capillary formation, and has specific mitogenic and chemotactic effects on vascular endothelial cells.[11] Studies carried out in colorectal cancer patients have shown that their preoperative serum VEGF-A levels are significantly higher than in normal controls.[14,15] Moreover, patients with advanced disease, ie, lymph node and/or distant metastasis, have significantly higher levels of preoperative serum VEGF-A,[16,17] and several groups have reported that serum VEGF-A is an independent prognostic factor in predicting the outcome of colorectal cancer.[15,18,19] VEGF-A expression in colorectal tissue, determined immunohistochemically, has also been found to be a predictive marker of the response of colorectal cancer to preoperative radiotherapy[20] and adjuvant chemotherapy.[21]

A relationship between the concentration of VEGF-A in serum or cancer tissue and the progression of disease has been reported. This relationship is seen in patients with breast, gastric, hepatocellular, lung, and colorectal cancer.[22–25]

In this study, we assessed a possible role for preoperative serum VEGF as a predictor of prognosis in colorectal cancer patients undergoing surgery with curative intent. An important biomarker would be of more benefit to specific subgroups of patients, namely Stage II and Stage III patients, than the existing systems and serum tumor markers, such as CEA. The aim of the present study was to determine whether serum VEGF could provide prognostic information independent of CEA in patients undergoing curative surgery.

Materials and Methods

Patients

One hundred and fifty-eight patients who underwent resection of colorectal carcinoma at our institution

between September 2000 and September 2005 were enrolled in this retrospective study. Healthy samples obtained from 100 normal healthy volunteers were used as controls. The absence of disease such as infection and asymptomatic early adenocarcinoma or adenoma was confirmed by clinical history, physical examination, routine biochemical tests, including liver and renal function tests, and colonoscopy. The patients included 99 men and 59 women with a mean age of 60 (range 30–78) years. None of the patients had had chemotherapy or neoadjuvant radiochemotherapy. Locations of the tumors and distant metastases were determined by barium enemas, colonoscopies, computed tomography, and magnetic resonance imaging. The primary lesion was located in the rectum in 55 patients, the sigmoid colon in 61, the ascending colon in 22, the transverse colon in 11, and the descending colon in nine. Eighteen patients were diagnosed as having synchronous liver metastasis and eight patients were diagnosed with both liver metastasis and peritoneal dissemination. Tumor resection was performed in all patients and simultaneous partial hepatectomy for liver metastases was performed in 20 patients. No preoperative mortality was observed among these patients. Eighty patients had poorly differentiated adenocarcinoma, whereas adenocarcinoma was well or moderately differentiated in 140 patients. All patients were classified according to the Union for International Cancer Control stage classification using resected specimens. There were 24 patients with Stage I disease, 59 patients with Stage II disease, and 57 patients with Stage III disease. Eighteen patients with distant metastases were classified as having Stage IV disease. Stage III and IV patients received fluorouracil-based chemotherapy, whereas no postoperative adjuvant therapy was given in Stage I and II patients. Patients were observed at three-monthly intervals for 24 months after completion of surgery, then every six months for three years, and yearly thereafter. Patient anamnesis and physical examination was done at each visit, and chest x-ray, colonoscopy, and computed tomography were performed once per year. The median follow-up duration was 65 (mean 50.2 ± 19.7) months. Of 158 patients studied, 70 died as a result of primary or recurrent disease. The clinicopathologic parameters studied for their prognostic value were tumor size, tumor classification, vessel involvement, lymphatic invasion, lymph node metastases, distant metastases, and serum concentration of CEA.

Serum and tissue protein assays

Peripheral venous blood samples were obtained from all 158 patients before surgery. Serum samples obtained from 100 normal healthy age-matched volunteers were used as controls. The absence of disease was assessed by clinical history, physical examination, and routine laboratory tests, including liver and renal function tests. Serum samples were allowed to clot, and were then stored at −80 °C until use. All dissected tissue specimens were cut into 5 mm cubic blocks, snapfrozen in liquid nitrogen immediately, and stored at −80 °C. VEGF levels in cancer tissue and adjacent normal tissue were analyzed in 89 of the 158 patients. These specimens were homogenized and tissue extracts were obtained. Before collection of serum and tissue extracts from the patients and healthy controls, their informed consent was obtained for the use of samples in future experiments. VEGF concentrations were quantified by a Quantikine® human VEGF immunoassay (R & D Systems Inc, Minneapolis, MN). The serum samples were incubated overnight at 4 °C on microtiter plates coated with a murine monoclonal antibody against human VEGF. Unbound proteins were washed off, and an enzyme-linked polyclonal antibody specific for VEGF is added to "sandwich" the VEGF immobilized during the first incubation. A horseradish peroxidase substrate solution was added, and color was developed in proportion to the amount of antibody-bound VEGF. Color absorbance was read at 450 nm. VEGF concentrations were expressed as pg/mg. Protein concentration was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL). The lower limit of detection for serum VEGF concentration was 0.01 pg/mL. Tissue concentrations were expressed as pg/mL/protein. CEA concentrations were determined by enzyme immunoassay.[26]

Statistical analysis

Data are presented as means ± standard deviations. Comparisons were performed using the nonparametric Mann-Whitney U test for continuous variables and the Chi-squared test for categorical data. Correlations were analyzed by Spearman's coefficient analysis. Analyses of receiver-operating characteristics were performed to calculate cutoff values. Survival probabilities were

calculated using Kaplan-Meier product limits, considering treatment-related deaths and deaths caused by colorectal cancer. Differences between the two groups were determined using the log-rank test. The influence of each significant predictor identified by log-rank test was assessed by multivariate analysis using Cox's proportional hazards model. Two-sided *P* values of <0.05 were considered statistically significant.

Results

Serum VEGF and clinicopathologic characteristics of colorectal cancer

Serum VEGF levels were analyzed in 158 colorectal cancer patients and 100 normal controls. There were no age or gender differences between the two groups. We found lower serum VEGF levels in the control population than in patients with colon cancer (21.6 ± 9.1 pg/mL, $P < 0.0001$). VEGF concentrations in patients ranged from 20.2 to 105.5 pg/mL. Mean serum VEGF concentration in patients was significantly higher than in controls ($P < 0.0001$).

Table 1 shows the relationship between serum VEGF levels and clinicopathologic variables in patients and controls. Serum VEGF was associated with factors reflecting disease progression, including tumor size $>$ 41 mm ($P = 0.0001$), lymph node involvement ($P = 0.001$), and the presence of distant metastases ($P < 0.0001$). In addition, serum VEGF levels increased significantly in accordance with the progression of Union for International Cancer Control stage classification ($P < 0.0001$). To examine the predictive value of serum VEGF for different clinicopathologic characteristics, we conducted Chi-squared and Mann-Whitney U tests. We defined elevated serum VEGF levels according to the best predictive values calculated on receiver-operating characteristic analyses for tumor size $>$ 41 mm (25 pg/mL), lymph node metastasis (47.4 pg/mL), and distant metastasis (47.9 pg/mL), and used the criteria of 47.9 pg/mL for analyses of other parameters. An elevated serum VEGF level was associated with advanced disease (Stage III and IV, $P = 0.0001$), tumor size $>$ 41 mm ($P < 0.0001$), and metastasis ($P < 0.0001$).

Table 1. Relationship between serum VEGF levels and clinicopathologic factors in 158 patients with colorectal cancer.

Variable	n	VEGF (pg/mL)	*P*
Gender			
Male	95	75.9 ± 29.2	
Female	63	78.3 ± 20.6	0.49†
Age (years)			
<65	80	91.4 ± 30.1	
≥65	78	89.5 ± 25.7	0.21†
Tumor size (mm)			
<41 mm	79	33.8 ± 10.2	
≥41 mm	79	95.7 ± 23.1	0.0001†
Lymph node metatstasis			
N0	88	67.0 ± 24.5	
N1–3	70	98.3 ± 21.6	0.0001†
Distant metastasis			
M0	122	56.4 ± 21.8	
M1	36	89.7 ± 19.3	0.0001†
UICC classification			
I	21	31.0 ± 12.7	
II	61	57.8 ± 20.5	
III	48	83.1 ± 23.9	
IV	18	99.5 ± 15.2	0.0001*
Normal serum			
Levels	50	29.8	
All patients	158	90.5	0.0001

Notes: †Mann-Whitney U test; *Kruskal-Wallis analysis.
Abbreviations: UICC, Union for International Cancer Control; VEGF, vascular endothelial growth factor.

Serum VEGF and survival according to CEA levels

In our colorectal cancer patient population, we defined elevated serum VEGF and CEA levels according to the best predictive values calculated in the receiver-operating characteristic analyses, which found the best pair of values for highest sensitivity and highest specificity using a peak for each cutoff point. Patients with elevated serum VEGF and CEA levels had significantly poorer prognosis than those whose levels were below the cutoff value (log-rank test, VEGF, $P < 0.0001$; CEA, $P < 0.0001$, respectively). On the basis of Cox univariate proportional hazards analysis, advanced Union for International Cancer Control stage (III and IV, $P < 0.0001$), tumor size (>41 mm, $P = 0.0008$), lymph node metastasis ($P < 0.00019$), distant metastasis ($P < 0.0001$), elevated serum CEA levels ($P < 0.0001$), and elevated serum VEGF levels ($P < 0.0001$) were significant prognostic factors for poor overall survival. By multivariate analysis, distant metastasis ($P < 0.0001$) and elevated serum VEGF ($P < 0.0001$) were only the independent risk factors for predicting poor prognosis (Table 2). Figure 1 shows a scattergram of VEGF expression in cancer tissues and normal tissues.

Table 2. Univariate and multivariate analyses for prediction of survival in colorectal cancer.

Variables	Univariate (all patients)		
	HR	**95% CI**	***P* value**
UICC classification			
(III/IV versus I/II disease)	8	3.12–12.8	0.0001
Tumor size (≥ or <41 mm)	3.8	2.5–5.12	0.001
Lymph node metastasis			
(yes versus no)	4.12	3.0–6.15	0.0001
Distant metastasis			
(yes versus no)	15.2	9.81–19.7	0.0001
VEGF (≥ or <25.0)	3.9	2.1–5.5	0.0001
CEA (≥ or <3.5)	4.5	2.51–6.9	0.0001
	Multivariate (all patients)		
UICC classification			
(III/IV versus I/II)	0.77	0.23–2.13	0.75
Tumor size (≥ or <41)	1.44	0.81–2.80	0.49
Lymph node metastasis			
(yes or no)	2.09	1.01–4.96	0.17
Distant metastasis			
(yes or no)	16.73	11.15–27.5	0.0001
VEGF (≥ or <25.0)	3.0	1.53–6.21	0.0001
CEA (≥ or <3.5)	2.24	1.39–4.38	0.41

Abbreviations: UICC, Union for International Cancer Control; VEGF, vascular endothelial growth factor; CEA, carcinoembryonic antigen; HR, hazards ratio; CI, confidence interval.

Serum VEGF/CEA and survival in potentially curative disease

In Stage II and III disease, we defined elevated serum VEGF and CEA levels according to the best pair of values for highest sensitivity and highest specificity using a peak cutoff point for each. Survival curves for Stage II and Stage III patients, subdivided on the basis of serum VEGF > 25.2 pg/mL and CEA > 3.5 ng/mL are shown in Figures 2A, 2B, 2C, and 2D. Figures 2A and 2B show serum VEGF levels for Stage II and Stage III patients, respectively, and Figures 2C and 2D show serum CEA levels for Stage II and III patients, respectively. Elevated serum VEGF and CEA levels were associated with a poor prognosis in patients with Stage II and III disease ($P < 0.0001$ and $P = 0.03$, respectively). On the basis of Cox univariate proportional hazards analysis, tumor size > 0.41 mm ($P < 0.01$), lymph node metastasis ($P = 0.04$), elevated serum CEA ($P = 0.03$), and elevated serum VEGF ($P < 0.0001$) were significant prognostic factors for poor overall survival. By multivariate analysis, elevated serum VEGF ($P = 0.01$) was the only independent risk factor predicting poor prognosis ymph node metastasis. Furthermore, elevated serum VEGF was associated with poor survival (Stage II and Stage III

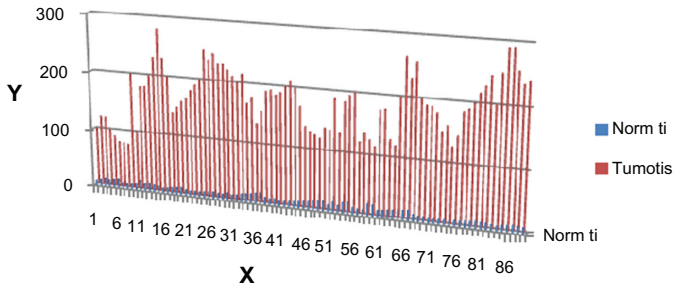

Figure 1. Vascular endothelial growth factor levels (pg/mL/mg protein) in tumor tissues and normal mucosa.*
Notes: *Blue: tissue levels in controls; red: tissue levels in patients. X axis: VEGF levels pg/ml/mg protein; Y axis: The number of patients.

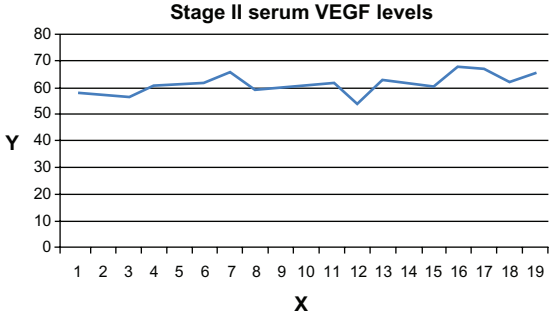

Figure 2A. Survival curves for Stage II patients according to vascular endothelial growth factor levels.
Notes: X axis: Serum VEGF levels; Y axis: Number.
Abbreviation: VEGF, vascular endothelial growth factor.

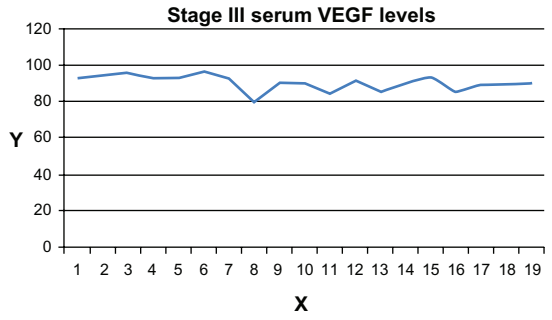

Figure 2B. Survival curves for Stage III patients according to vascular endothelial growth factor levels.
Notes: X axis: Serum VEGF levels; Y axis: Number.
Abbreviation: VEGF, vascular endothelial growth factor.

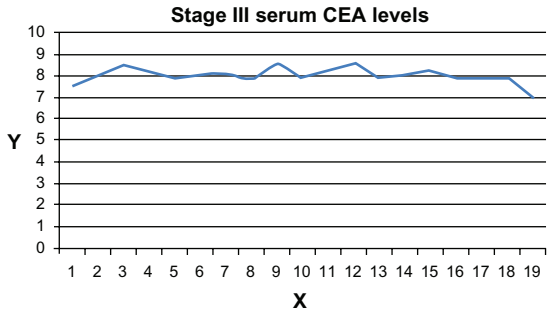

Figure 2D. Survival curves for Stage III patients according to carcinoembryonic antigen levels.
Notes: X axis: Serum CEA levels; Y axis: Number.
Abbreviation: CEA, carcinoembryonic antigen.

patients, $P = 0.003$ and $P = 0.005$, respectively) and was the only independent prognostic factor in Stage II or Stage III patients (Stage II and Stage III, $P = 0.001$ and $P = 0.03$, respectively).

Discussion

The clinical and pathologic staging of colorectal cancer after surgery remains the standard for clinical outcome. However, these methods do not accurately predict the clinical outcome for individual patients. A number of studies have investigated the use of various combinations of molecular markers to predict clinical outcome in the hope that these may help to identify high-risk patients and hence enable appropriate neoadjuvant and/or adjuvant treatment.[27–30]

In the present study, we showed that increased preoperative circulating VEGF levels were significantly correlated with tumor size, and nodal and distant metastases, which are well known prognostic factors. VEGF is a multifunctional cytokine that increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis, and many studies have shown that VEGF expression is increased in patients with cancer.[15–20] Previous research has shown that serum CEA levels are elevated in Stage III and IV patients, but not in Stage I or II patients.[19] In this study, we found no correlation between serum VEGF and CEA levels in colorectal cancer patients. These findings support our hypothesis that VEGF and CEA are independently regulated.

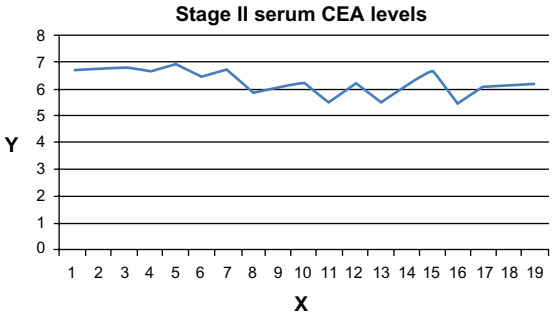

Figure 2C. Survival curves for Stage II patients according to carcinoembryonic antigen levels.
Notes: X axis: Serum CEA levels; Y axis: Number.
Abbreviation: CEA, carcinoembryonic antigen.

The Union for International Cancer Control staging system provides the most reliable information on prognosis, and is certainly useful for discriminating between patients with early-stage disease and those with advanced disease. However, its prognostic ability in patients with intermediate levels of tumor invasion is less accurate. Therefore, identification of sensitive prognostic markers would allow the use of postoperative adjuvant therapy in a subset of patients having a worse prognosis, with improvement in survival. Our study has shown that the preoperative serum concentration of VEGF was the only pretreatment prognostic factor in Stage II and III colorectal cancer patients. The ability to identify Stage II patients with a poor prognosis and in need of treatment to prevent recurrence could improve cancer survival rates. From a clinical point of view, classification of patients with Stage III tumors is also important, because intensive adjuvant chemotherapy and/or treatment with oxaliplatin could improve their survival rates.[20] Interestingly, serum VEGF was a stronger prognostic factor than lymph node metastasis in Stage II and III patients, who are routinely offered postoperative adjuvant chemotherapy. Kwon et al reported that increased VEGF concentrations in colorectal cancer patients are an important prognostic factor when assessing life expectancy.[31] Altomare et al also suggested that

VEGF concentrations are an independent risk factor for colorectal tumor recurrence.[3]

Conclusion

Preoperative serum VEGF may be a better prognostic marker in colorectal cancer patients, especially in those with Stage II and/or III disease, than the prognostic values of the Union for International Cancer Control staging system and CEA levels.

Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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