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World J Gastroenterol 2003;9(6):1227-1230
World Journal of Gastroenterology
 ISSN 1007-9327

• COLORECTAL CANCER •

Clinical significance of vascular endothelial growth factor expression and neovascularization in colorectal carcinoma

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Received: 2003-03-02 **Accepted:** 2003-04-01

Abstract

AIM: To clarify the association of vascular endothelial growth factor (VEGF) and microvascular density (MVD) expression with the angiogenesis and prognosis of colorectal cancer.

METHODS: A total of 97 cases of colorectal carcinomas were examined by immunohistochemical staining (SP method), using anti-VEGF and anti-factor $CD34^+$ monoclonal antibodies.

RESULTS: VEGF positive staining was obtained in 68 out of 97 cases (70.1 %), and observed mainly in the cytoplasm of tumor cells, and also frequently in stromal cells. VEGF expression was more intense in poorly differentiated adenocarcinoma in comparison with others, but there was no significant correlation between VEGF expression and age, sex and stage. A significant correlation was found between the MVD and grades, and there was no significant relationship between the MVD and age, sex, and stage. The MVD in the VEGF positive group (68 cases) was higher than that in the negative group. Upon multivariate analysis, the significant variables were stage, tumor grade and MVD; VEGF expression was not an independent prognostic factor.

CONCLUSION: The expression of VEGF has a significant correlation with MVD; MVD expression has prognostic value but VEGF has not in colon cancer.

Zheng S, Han MY, Xiao ZX, Peng JP, Dong Q. Clinical significance of vascular endothelial growth factor expression and neovascularization in colorectal carcinoma. *World J Gastroenterol* 2003; 9(6): 1227-1230
http://www.wjgnet.com/1007-9327/9/1227.asp

INTRODUCTION

Angiogenesis is an essential process required for the growth and metastatic ability of solid tumors[1]. Some studies demonstrated that an increase in microvascular density (MVD) was found to be closely associated with the expression of vascular endothelial growth factor (VEGF), and that MVD and VEGF expression had a prognostic value in predicting metastasis of various malignant solid tumors[2,3]. Several studies have noted that the level of VEGF expression, a strong angiogenic factor, correlates with neovascularity and tumor progression in human breast and brain cancers and experimental tumor models[4,5]. In this study, we investigated the correlation of the VEGF and MVD in the tumor tissue of patients with colon cancer.

MATERIALS AND METHODS

Patients and tumor specimens

Tumor specimens from 97 patients resected for colorectal cancers, from the Second Affiliated Hospital of Zhejiang University (Hangzhou, China) from March 1993 to September 1995 were assessed. The age of the patients ranged from 36 to 74 years; 58 were male and 39 were female; average age, male 57.5 years old, female 61.5 years old. The patients were staged according to operation and pathological findings with UICC TNM classification: 9 (9.3 %) in stage I, 38 (39.2 %) in stage II, 32 (32.9 %) in stage III, and 18 (18.6) in stage IV.

Immunohistochemistry

Specimens were fixed in a 10 % formaldehyde solution and embedded in paraffin. Sections, 5 μm thick, were cut and mounted on glass slides. Immunohistochemical staining was performed using the avidin-biotin method. Staining for VEGF was performed using an anti-VEGF monoclonal antibody (MAb) (Calbiochem, Cambridge, UK). Staining for vascular endothelial cells was performed using an anti-CD34 MAb (DAKO, Copenhagen, Denmark). Briefly, formalin-fixed, paraffin-embedded 5 μm tissue sections were deparaffinised with xylene, dehydrated in ethanol and incubated with 3 % hydrogen peroxidase for 5 min. After washed with phosphate-buffered saline (PBS), tissue sections were incubated in 10 % normal bovine serum for 20 min, followed by an overnight incubation with anti-VEGF (1:50) antibody or anti-CD34 antibody (1:50). Biotinylated goat antimouse and antirabbit immunoglobulins were used as secondary antibodies. Peroxidase-conjugated avidin was used as a dilution of 1:500. Finally, 0.02 % diaminobenzidine and 1 % hydrogen peroxide in PBS were used as the substrate. Normal mouse IgG diluted to an equivalent protein concentration was used as a control in place of the primary antibody. Counterstaining was performed with haematoxylin.

Any single brown-stained cell that indicates an endothelial cell stained with CD34 was counted as a single vessel. Branching structures were counted as a single vessel, unless there was a break in the continuity of the structure. The stained sections were screened at 5 times magnification, to identify the areas of highest vascular density. After the area of highest neovascularization was identified, individual vessel counts were performed at ×200 magnification.

Evaluation of VEGF expression

For the evaluation of VEGF expression, immunostaining was classified in two groups, corresponding to the percentage of immunoreactive cells; the cut-off point to distinguish low from high VEGF expression was 25 % of positive carcinoma cells.

Statistical analysis

Statistical comparisons for significance were made with the Student's *t* test and χ^2 test. Multivariate analysis was performed

using the Cox' s regression multiple hazard model. $P<0.05$ was considered statistically significant.

RESULTS

VEGF expression

Positive staining was obtained in 68 out of 97 cases (70.1 %) and a typical immunohistochemical staining is shown in Figure 1. VEGF immunoreactivity was observed mainly in the cytoplasm of tumor cells, and also frequently in stromal cells. The distribution of VEGF-staining was not continuous in the whole slide of the specimen. VEGF expression was more intense in poorly differentiated adenocarcinoma in comparison with other tumors ($P=0.014$), but there was no significant correlation between VEGF expression and age, sex and stage (Table 1).

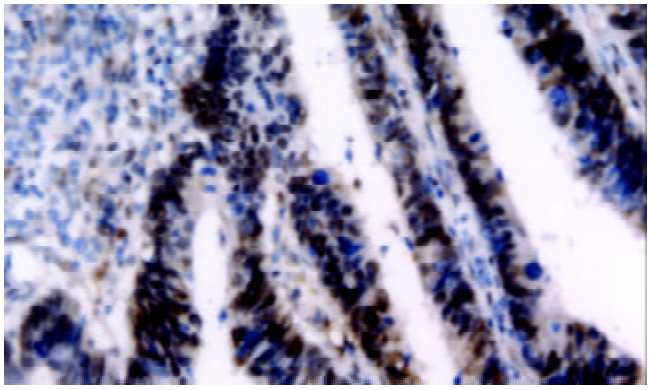

Figure 1 VEGF expression in colorectal cancer specimen. VEGF immunoreactivity was observed mainly in the cytoplasm of tumor cells, and also frequently in stromal cells.

Table 1 Relationship between clinicopathologic factors and VEGF expression ($n=97$)

Variables	*n* (%)	VEGF expression		
		+ *n*(%)	- *n*(%)	*P* value
Age				
<45 years	36	26(72.2)	10(27.8)	
>45 years	61	42(68.8)	19(31.2)	
Sex				
Male	58	41(74.1)	17(25.9)	
Female	39	27(68.9)	12(31.1)	
Different differentiation				
Well	28	15(51.1)	13(48.9)	
Moderate	36	26(72.2)	10(27.8)	
Poor	33	27(81.8)	6(18.2)	0.014
Stage				
I	9	7(77.8)	2(22.2)	
II	38	27(71.1)	11(28.9)	
III	32	23(72.2)	9(27.8)	
IV	18	11(61.5)	7(38.5)	

$P<0.05$ *vs* Well differentiated group.

Microvascular density (MVD)

Any single brown-stained cell that indicates an endothelial cell stained with CD34 was counted as a single vessel (Figure 2). The median MVD was 187.6±17.3. A significant correlation was found between the MVD and different grade (0.028), and there was no significant relationship between the MVD and age, sex, and stage (Table 2).

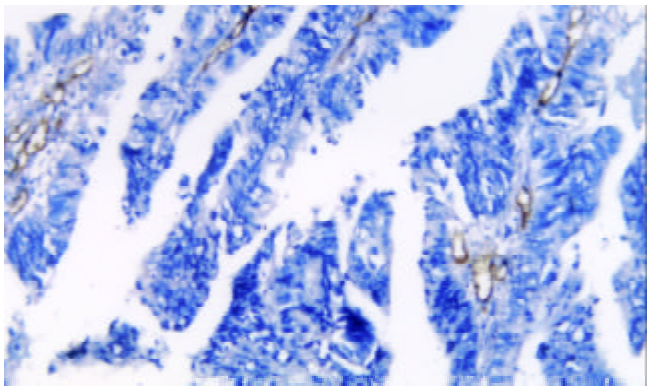

Figure 2 Microvascular density in colorectal cancer specimen. The single brown-stained cell indicates an endothelial cell that was stained for the presence of CD34.

Table 2 Relationship between clinicopathologic factors and MVD ($n=97$)

Variables	*n*	MVD		
		high MVD	low MVD	*P* value
Age				
<45 years	36	20(55.6)	16(44.4)	
>45 years	61	33(54.3)	28(45.7)	
Sex				
Male	58	32(55.2)	26(44.8)	
Female	39	21(53.8)	18(46.2)	
Different differentiation				
Well	28	12(42.9)	16(57.1)	
Moderate	36	20(55.6)	16(44.4)	
Poor	33	21(63.6)	12(36.4)	0.028
Stage				
I	9	5(55.6)	4(44.4)	
II	38	20(52.6)	18(47.4)	
III	32	18(56.3)	14(43.7)	
IV	18	10(55.6)	8(44.4)	

$P<0.05$ *vs* Well differentiated group.

Table 3 Relationship between MVD and VEGF expression

Variable		MVD	*P* value
VEGF	(+)	213.4±12.8	
Expression	(-)	138.7±19.4	0.027

$P<0.05$ *vs* VEGF (-) group.

Table 4 Multivariate analysis of overall survival by Cox proportional hazards model

Variable	Categories	Hazard ratio	SEM	*P* value
Age	>45 years versus <45 years	1.877	0.3796	
Sex	Male versus female	1.216	0.4071	
Differentiation	Poor versus others	2.361	0.2438	0.0217
Stage	I, II versus III, IV	2.973	0.1976	0.0012
VEGF	(+) versus (-)	1.164	0.3874	
MVD	>187.6 versus <187.6	2.526	0.2126	0.0314

$P<0.05$ *vs* Stage I, II group.

Association between VEGF expression and MVD

The MVD in the VEGF positive group was 213.4±12.8, and that in the negative group was 138.7±19.4. MVD in the VEGF

positive group was higher than that in the negative group (P=0.027) (Table 3).

Multivariate analysis

Upon multivariate analysis of all patients, the significant variables were stage, tumor grade and MVD. Age, sex, VEGF expression were not independent prognostic factors (Table 4).

DISCUSSION

Microvascularity is important in cancer growth and metastasis because it is involved in the transport of various nutrients to the tumor cells[1,2]. In this process of tumor growth and angiogenesis numerous angiogenic factors are involved. In recent years several of these factors have been identified[3-6]. One of the most important regulators of angiogenesis is VEGF. It induces the vascular stroma not only as a direct endothelial cell mitogen, but also as a potent mediator of microvessel permeability. This ability of VEGF to induce fenestrations on microvessels has been demonstrated in experimental tumors[7,8].

The VEGF is overexpressed in a variety of benign tissues and malignant human tumors. The expression of VEGF suggests that it plays a role in luminal secretion by increasing local vascular permeability[9]. In neuroendocrine tumors the high levels of VEGF expression could indicate a role for VEGF in the release of gastrointestinal hormones through the regulation of baseline permeability of the normal microcirculation[10]. The high level of VEGF expression in some malignant tumors, such as breast cancer, non-small cell lung cancer, bladder cancer and gastric cancer, is a characteristic feature of these tumors. Several studies have demonstrated that high microvessel density is a useful indicator for poor prognosis in these cancers[11-15].

CD34 antigen is expressed on immature human haematopoietic precursor cells and is progressively lost during maturation[16,17]. In normal resting tissues, anti-CD34 antibodies are predominantly neated with the luminal endothelial membrane, whereas the abluminal membrane is negative or only weakly positive. In contrast, significant staining of the endothelial abluminal microprocesses (EAM) has been found in tumor stroma[18]. It has been shown that CD34 is a marker for EAM during angiogenesis and the antigenicity of CD34 is preserved by freezing, or ethanol, and formalin fixation[19].

Microvessel density (MVD) and expression of VEGF act as a highly specific inducer of angiogenesis. Strong VEGF expression and high MVD are considered important parameters of tumor angiogenesis and related to poor survival probability in vulvar cancer patients[20,21]. In primary breast cancer, MVD and VEGF serve as a parameter for determining tumor biological, metastatic potential and prognosis[22]. VEGF is highly related to angiogenesis of gastric carcinoma and promotes growth, invasion and metastasis of gastric carcinoma, VEGF expression and MVD are predictors for the biological behavior of gastric carcinoma[21,23]. In primary liver cancer, besides tumor stage, satellite nodules and portal vein embolus, the MVD and VEGF expressions are also of prognostic significance[24]. Intense VEGF staining was found in the majority of advanced primary SCCs, lymph node metastases, and human SCCs in severe combined immunodeficent mice, where no dysplasia, CIS, or early SCCs showed intense immunostaining. Suggesting a role for VEGF in both clinical and experimental HNSCC[25,26]. By univariate analysis, VEGF expression and MVD in the biopsy specimens were significant predictors of bladder cancer recurrence. By multivariate analysis, only VEGF expression was an independent prognostic factor[27]. The metastatic potency of NPC tissue and the prognosis of the patients with NPC could be estimated by measuring MVD and the expression of VEGF in NPC tissue[28]. Previously it was demonstrated that in prostate tumors, angiogenesis measured as microvessel density (MVD) was associated with tumor stage as well as WHO grade and was an independent predictor of clinical outcome. Vascular endothelial growth factor (VEGF) is a major inducer of angiogenesis[29]. The relationship between VEGF expression and MVD in ovarian carcinoma suggests that in conjunction with the established clinicopathologic prognostic parameters of ovarian carcinoma, VEGF expression may enhance the predictability of patients at high risk for tumor progression who are potential candidates for further aggressive therapy[30]. But MVD in synovial sarcomas did not correlate with prognosis or VEGF expression, angiogensis in synovial sarcoma might be controlled by angiogenesis activators other than VEGF[31]. In patients with invasive cervical cancer VEGF expression has no prognostic value in contrast with the MVD[32].

In this study, we found positive VEGF staining was obtained in 68 out of 97 cases (70.1 %), VEGF immunoactivity was observed mainly in the cytoplasm of tumor cells, and also frequently in stromal cells. VEGF expression was more intense in poorly differentiated adenocarcinoma in comparison with other tumors, but there was no significant correlation between VEGF expression and age, sex and stage. A significant correlation was found between the MVD and different grade, and there was no significant relationship between the MVD and age, sex, and stage. MVD in the VEGF positive group was higher than that in the negative group. Upon multivariate analysis, the significant variables were stage, tumor grade and MVD; VEGF expression was not an independent prognostic factor. We conclude that MVD but not VEGF expression has prognostic value in colon cancer.

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Edited by Zhang JZ