Evaluation of Angiogenesis with the Expression of VEGF-C and CD34 in Human Colon Cancer

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Abstract: Background: The most potent angiogenic factor is the vascular endothelial growth factor (VEGF), and CD34 is an endothelial antigen that has been used to highlight the microvasculature vessel density (MVD) as a direct marker of the degree of neoangiogenesis. In the present study we report that VEGF expression and its relationship with MVD, in poorly and well differentiated colon adenocarcinoma, in order to consider the possibility of using the correlation between both antibodies as a prognostic factor. Materials and Methods: Tumor sections were stained by immunohistochemistry for CD34 and VEGF. Results: The mean of VEGF and CD34 did not show any significant differences between both types of tumors. Conclusion: The conventional factors taken into consideration were not significantly related to the angiogenic factors examined, so we could affirm that the correlation of both markers could be useful as prognostic factor.

Keywords: Colon cancer, VEGF, CD34, angiogenesis, colon, adenocarcinoma.

INTRODUCTION

In colorectal cancer, neovascularization is likely to be an important stept in the transition from a latent to a metastatic state, which is associated with a poor prognosis.

Interaction between tumor cells and their supportive stroma plays a crucial role in tumor development and progression [1], in which angiogenesis is the essential process due to the fact that solid tumors cannot grow beyond 1-2 mm in diameter without neovascularization [2].

VEGF it is a glycoprotein with angiogenic, mitogenic and vascular permeable enhancing activity in endothelial cells [3] and is the most potent angiogenic factor known [4].

The VEGF family includes VEGF-A, B, C and D, as well as PLGF (placenta growth factor). In humans, VEGF-C and D activate VEGFR-2 and VEGFR-3 receptors, both of which are essential for vascular development [5,6]. Interestingly, in various human cancer, a positive correlation between VEGF-C, and VEGFR-3 expressions was observed in the primary tumor with lymphatic invasion and lymph node metastasis [7-9].

The CD-34 is an endothelial antigen that has been used to highlight the microvessel density (MVD) as a direct marker of degree of neoangiogenesis; however, it can react not only with "newly forming" vessels but also with normal vessels just trapped within the tumor tissues [10]. Since 1991, when Weidner N [11] showed that assesing tumor MVD could be useful in evaluating the agressiveness of breast carcinoma, several studies have found similar results for other types of malignancies [12].

In the present study we report the VEGF-C expression and its relationship with the MVD in poorly and well-differentiated adenocarcinoma, using immunohistochemistry with anti-VEGF and anti-CD34, in order to consider the possibility of using the correlation between both antibodies as a prognostic factor.

MATERIALS AND METHODS

Tumor

Tumor specimens from 54 patients (35 males and 19 females, mean age 60 +- 10 years old) who had undergone potentially curative surgical resections for primary sporadic colorectal adenocarcinoma, treated at the Hospital Interzonal Especializado de Agudos y Crónicos San Juan de Dios, La Plata, were analyzed for VEGF-C and CD34 expression.

Determination of VEGF and CD34 Expression

Desparaffinized and rehydrated sections were microwaved for X mM in citrate buffer, pH 6. Endogenous peroxidase was blocked for 20 minutes. The primary antibody against VEGF-C1 (mouse monoclonal antibody; Santa Cruz Biotechnology, California, USA 1:100 dilution) and a mouse monoclonal antibody against CD34 protein (Santa Cruz, Biotechnology, California, USA 1:80 dilution) were incubated for 60 minutes. Bound primary antibody was detected by Envision System (Dako) for 30 minutes and the reaction was developed using diaminobenzidine, and was counter stained with Mayer hematoxylin. The positive control was a section of lung tumor that had previously shown to have a high

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VEGF and CD34 content, by immunohistochemistry. VEGF and CD34 stains were seen in the tumor cell cytoplasm.

The expression of VEGF was assessed according to the percentage of immunoreactive cells in a total of 1000 neoplastic cells (quantitative analysis). There was > 95% agreement between the two observers for the VEGF evaluation. A final score was determined by consensus after re-examination. MVD was assessed using the criteria of Weidner *et al.* [11]. The areas of highest vascularization were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD34. Any brown stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel. Each count was expressed as the highest number of microvessels identified within 0.3 mm^2 fields at a magnification of 200 X. Ten fields of the most intense vascularization (hotspots) were analyzed for each tumor. Two investigators performed all counts simultaneously, and both had to agree on what constituted a single microvessel before a vessel was included in the count.

Statistical Analysis

The statistical analysis of VEGF expression was performed using Student t-test, because the distribution of samples was normal. The marked tumoral cells were recorded by counting 50 areas, and the total nuclei every 10 areas. The results were expressed as a percentage of marked cells.

The CD34 expression was analyzed by Student t-test and was expressed as mean ± SE of the total of marked vessels in the selected areas for every tumor.

RESULTS

This study was realized with 55 patients, 32 were male and 23 female. Most of them were more than 60 years old, were in stage III, and had a well differentiated adenocarcinoma. (Table **1**).

High expression of VEGF was seen in 22 patients (95.6 %) with poor differentiated adenocarcinomas, and in 26 (81.2 %) with well differentiated adenocarcinomas. A high expression of CD34 was seen in 15 patients (65.2 %) with poor differentiated adenocarcinomas, and in 19 (59.3 %) with well differentiated adenocarcinomas. (Table **2**).

We could observe that the expression of both analyzed factors, VEGF and CD34, did not show any significant difference between both types of tumor. (Table **3**).

At the end of our study, one patient died at the age of 37, with a poor differentiated adenocarcinoma that evidenced the presence of positive lymph nodes.

DISCUSSION

Angiogenesis is a complex process that involves endothelial cell migration, capillary bredding, neovascular remodeling, in addition to endothelial cell proliferation [13]. The growth of solid tumors like those analyzed in this study, needs an adequate vascular network to remove waste and for

Table 1. Summary of all the Clinicopathological Factors of the Patients

		n or %
Sex	Male	32
	Female	23
Degree of differentiation	Well	59.2%
	Poor	42.6%
Age	< 60	13
	> 60	42
Stage	I	22% (12n)
	II	11% (6n)
	III	66.6% (36n)

n: number of patients
n total: 55 patients
%: percentage of tumors degree of differentiation

Table 2. Number of Patients with Colon Adenocarcinoma and their Immunohistochemical Parameters

Immunohistochemistry	**VEGF**		**CD34**	
	Poor differentiated	Well differentiated	Poor differentiated	Well differentiated
Strong	**22/23**	**26/32**	**15/23**	**19/32**
Low	**1/23**	**6/32**	**8/23**	**13/32**

The values were expressed as number of immunopositive patients / total of patients.

Table 3. Immunohistochemical Parameters of Angiogenic Factors and Conventional Risk Factors

	Poor	Well	p <
(n)	23	32	
VEGF (x)	34.7±3.2	40.8±3.1	ns
CD34 (x)	5.9±0.6	5.9±0.5	ns
Age more than 45	19	26	
Male	14	21	
Female	9	11	
Lymph nodes +	15	17	
Lymph nodes -	7	15	

Data were expressed as mean +/- standard error.
p: probability.
(n): number of patients.
(x): mean.
(ES): standard error.

the supply of oxygen and nutrients. It has also been established that tumor cell proliferation decreases with increading distances from the blood vessels [14,15].

Several authors have demonstrated that VEGF-C is expressed in several malignances including gastric [16,17], breast [18], thyroid [19], prostate [20], esophage [21] and colorectal carcinomas [22]. Moreover, Kawakami M. [23] suggested that a balance amomg VEGF-B,C and D might contribute to the lymphangiogenic process and metastasis in colorectal cancer.

Onogawa S. [24] has shown that VEGF-C is expressed heterogeneously in colorectal carcinoma and its expression suggests a poor prognosis. In concordance with that Kaio E. [25] showed that lymph node metastasis and VEGF expression were significant risk factors.

On the other hand, Zheng S. [26] observed that MVD but not VEGF expression, had a prognostic value in colon cancer. Our results may be consistent in part with this study, because as it was mentioned above, no differences were found in VEGF expression between both types of tumors, neither were any differences found in MVD expression. Furthermore, no significant differences were observed in patients with positive lymphatic nodes in both types of tumors.

All the patients were operated 3 years ago (2006), so it could be affirmed that the rate of survival was high because only one patient died, but strong conclusion was assumed regarding longevity.

In conclusion, VEGF and CD34 are excellent markers of neoangiogenesis. It is known that both tumoral cell types have the same clinical prognosis. This study showed that VEGF and CD34 have similar labeled behavior so, it may be assume that the markers pattern obtained here may be used as a malignance prognostic factor.

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