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Comparison of blood neoangiogenesis and lymphatic vascularization in colorectal adenomas from patients with and without concomitant colorectal cancer

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Blood and lymphatic vessel proliferation is essential for tumor growth and progression. Most colorectal carcinomas develop from adenomas (adenoma-carcinoma sequence) in a process due to accumulation of molecular genetic alterations. About 5% of adenomatous polyps are expected to become malignant, but data on the differential angiogenic patterns of these lesions in patients with and without concomitant cancer are missing. The aim of the present study is to compare the angiogenic and lymphatic patterns of adenomatous polyps from patients with and without sporadic cancer. Thirty adenomatous polyps (15 from patients with another principal malignant lesion, and 15 from patients without cancer) were submitted to immunohistochemical staining for CD105 (marker for neoangiogenesis) and D2-40 (marker for lymphatic endothelium). Microvessel density and total vascular area were determined by computer image analysis to quantify the immunostained and total areas, and to assess the number of microvessels. Adenomas from patients with carcinoma showed significantly higher values of total vascular area determined by immunostaining for CD105 (cutoff value = 4386 μm^2; P = 0.019) and of lymphatic microvessel density determined by immunostaining with D2-40 (cutoff value = 11.5; P = 0.041) when compared with those from patients without cancer. The present data indicate a significant increase in blood microvascular area and in lymphatic microvascular counts in adenomas removed from patients with cancer.

Key words: Angiogenesis; Colorectal adenoma; Colorectal cancer; Immunohistochemistry; CD105; D2-40

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Introduction

Colorectal carcinoma (CRC) represents an important cause of cancer mortality in industrialized countries. Most cases (80%) correspond to sporadic carcinomas and arise from colorectal adenomas (1). The adenoma-carcinoma sequence involves accumulations of genetic alterations causing progressive disorders in the cell cycle (2). About 5% adenomatous polyps will probably become malignant (3).

Angiogenesis plays an important role in tumor progression and metastasis in most human solid tumors (4-7). This fact has led to new perspectives in the research of prognostic indicators and of new therapeutic strategies. The fact that 20-30% of patients with CRC treated with potentially curative surgery succumb from recurrent disease suggests that the conventional prognostic factors are not

totally sufficient (8-11).

Most studies have evaluated angiogenesis as a potential prognostic or predictive factor in CRC both in early and advanced disease (12,13). Comparison of literature data has been frequently hindered by variations in patient management, such as indication of adjuvant therapy, and in the methods for analysis of angiogenesis (different immunohistochemical markers, quantification methods, parameters quantified, etc.) (12,14,15).

Endoglin (CD105) is a membrane glycoprotein, part of the TGF-beta receptor complex, involved in angiogenesis. Markers for this protein identify newly formed blood vessels, representing a helpful tool in the evaluation of neoangiogenesis (16). In CRC, CD105 has been correlated to prediction of metastasis (17).

D2-40 is a monoclonal antibody directed against the oncofetal antigen M2A, present in germ cells, lymphatic endothelium, and some neoplasms such as mesotheliomas (18,19). Using this antibody, it has been demonstrated that the colorectal mucosa indeed presents lymphatic vessels in normal, inflammatory and neoplastic conditions (20,21). However, in contrast to CD105, present in newly formed blood vessels, the presence of D2-40 does not indicate the degree of neolymphangiogenesis. Lymphatic vessel density assessed by D2-40 has been correlated with the prediction of metastasis and with a poor outcome of CRC (22-24).

In spite of the many reports on angiogenesis in cancer, data on the differential angiogenic patterns of adenomatous lesions in patients with and without concomitant CRC are not available. The purpose of the present study was to compare blood angiogenesis and lymphatic vessels between two groups of adenomatous polyps, one from patients with concomitant CRC at another site of the mucosa, and the other from patients without carcinoma, using microvessel counting and total vascular area determination with image analysis software. Our aim was to determine potential differences between adenomatous polyps from the two groups of patients, and weather the presence of carcinoma could influence the vascularization of colorectal adenomas.

Material and Methods

Tissue samples

A retrospective study was performed on 30 low-grade adenomatous polyps removed by endoscopy or surgery from 15 patients with sporadic CRC and 15 patients without carcinoma. The latter group did not show evidence of carcinoma from the time of the procedure throughout a 5-year follow-up. Hamartomatous and inflammatory polyps were excluded from the study.

The samples were selected from the files of the Department of Pathology, State University of Campinas Hospital, Campinas, SP, Brazil, and included patients diagnosed from 1987 to 2003. The group of patients with CRC consisted of 8 males and 7 females ranging from 33 to 82 years (median: 61 years); 5 cases were staged as I, 7 as II, 2 as III, and 1 as IV, according to the TNM pathological staging system (25). Only low-grade adenomatous polyps, which had been removed from the colorectal mucosa concomitantly to or soon after the diagnosis of the main malignant lesion, without the effect of neoadjuvant therapy, were included. There were 8 tubular, 6 tubulovillous, and 1 villous adenomas.

In the group of patients without a diagnosis of CRC 9 were males and 6 were females ranging from 20 to 82 years (median: 56 years). There were 11 tubular and 4 tubulovillous adenomas.

Immunohistochemistry

Tissue specimens had been fixed in 10% formalin and embedded in paraffin and 3-µm thick sections were placed on silanized slides. Endogenous peroxidase activity was quenched by incubating the slides with 3% H_2O_2 for 10 min. Antigen retrieval was achieved by microwaving tissue sections in 10 mM citrate buffer, pH 6.0, in four cycles of 5 min each. Sections were incubated at room temperature for 20 min with mouse monoclonal antibodies to CD105 (Endoglin, Clone SN6h, Dako, USA; diluted 1:15) and to D2-40 (Dako, diluted 1:400). Antigen-antibody binding was detected using the Advance system (Dako). Internal and external positive and negative controls were run concomitantly in each reaction batch.

Evaluation of immunohistochemistry

Digital images from two “hot spot” fields stained by each marker were captured. One area corresponded to the upper/inner portions of the lesions, and the other to the deeper area of the polyps. The upper/inner areas were grouped together because there were some small adenomas in which both areas appeared in the same image. Digitalization was done at 200X magnification, 120 dpi, using a digital camera (Leica DFC360 FX, Leica, Germany) connected to a bright field microscope (Leica DM5000 B).

The images were examined with image analysis software (Leica QWin Standard V3, Microsystem Imaging, Leica) set to detect color intensities in a fixed and constant range. Every image was evaluated using this standardized program to quantify the proportion between stained and total areas and to assess the number of microvessels.

Immunostained blood and lymphatic vessels were marked with a circle by the pathologist who analyzed the image to perform automated quantification. An example of the resulting image prepared for analysis after selection of the immunostained vessels is shown in Figure 1. This resulted in the evaluation of two parameters for each marker: microvessel density (MVD) and total vascular area (TVA).

Statistical analysis

Statistical analysis was performed using the SAS System for Windows software package (version 9.1.3). For the quantitative parameters, the minimum and maximum values, mean, standard deviation, and median were analyzed. For the qualitative variables, the absolute and relative frequencies were analyzed. The non-parametric Mann-Whitney test was used to compare two groups and the Kruskal-Wallis test was used for three or more groups. The Dunn comparison test was used for multiple comparisons. The level of significance was set at 5% in all analyses.

Results

CD105 in adenomas from patients with CRC

MVD ranged from 0 to 15 (median 5; mean 5.53) in "hot spots" of the upper/inner parts of adenomas and from 0 to 37 (median 1; mean 5.40) in the deeper areas. TVA ranged from 0 to 219905 μm^2 (median 7498; mean 25681.33 μm^2) in the "hot spots" of the upper/inner regions and from 0 to 27255 μm^2 (median 755; mean 6215.00 μm^2) in the deeper area.

CD105 in adenomas from patients without CRC

MVD ranged from 0 to 10 (median 2; mean 3.20) in "hot spots" of the upper/inner part of adenomas and from 0 to 10 (median 1; mean 2.53) in the deeper area. TVA ranged from 0 to 19660 μm^2 (median 183; mean 3810.20 μm^2), in the "hot spots" of the upper/inner parts and from 0 to 66522 μm^2 (median 143; mean 8114.40 μm^2) in the deeper area The results for CD105 are summarized in Table 1.

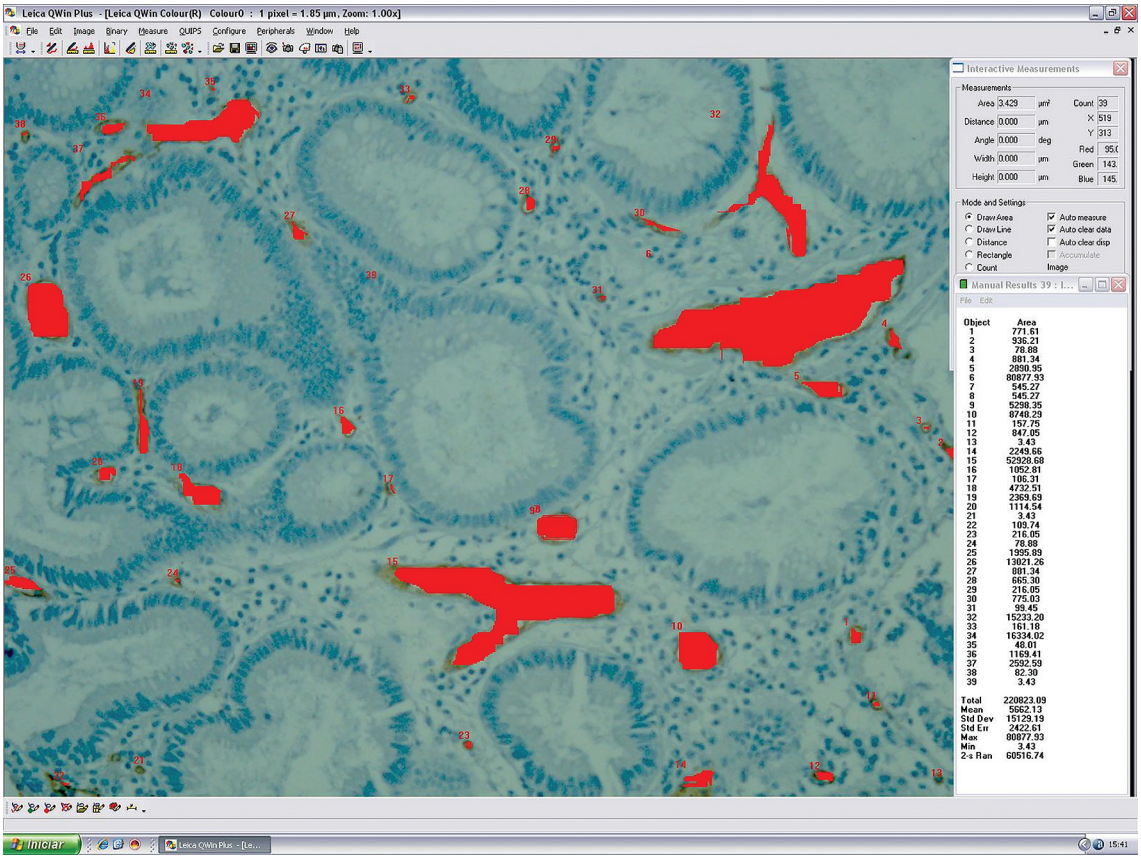

Figure 1. Evaluation of microvessels in immunostained sections by computer image analysis: the positive vessels are delimited in red and their area was calculated by software.

Table 1. Microvessel density (MVD; number of microvessels counted) and total vascular area (TVA, μm^2) determined by immunostaining for CD105 in the two areas (upper/inner and deeper) of adenomas in 15 patients with CRC and 15 patients with no CRC.

Variables	Area	Patient group	Range	Mean ± SD	Median
MVD	Upper/inner	CRC	0-15	5.53 ± 5.22	5
		No CRC	0-10	3.20 ± 3.41	2
	Deeper	CRC	0-37	5.40 ± 9.56	1
		No CRC	0-10	2.53 ± 3.27	1
TVA	Upper/inner	CRC	0-219905	25681.33 ± 55338.71*	7498
		No CRC	0-19660	3810.20 ± 5910.57	183
	Deeper	CRC	0-27255	6215.00 ± 9110.95	755
		No CRC	0-66522	8114.40 ± 17675.36	143

CRC = colorectal cancer; No CRC = no colorectal cancer; SD = standard deviation. *P < 0.05 compared to No CRC (non-parametric Mann-Whitney test).

Table 2. Lymphatic microvessel density (MVD; number of lymphatic microvessels counted) and total vascular area (TVA, μm^2) determined by immunostaining for D2-40 in the two areas (upper/inner and deeper) of adenomas in 15 patients with CRC and 15 patients with no CRC.

Variables	Area	Patient group	Range	Mean ± SD	Median
MVD	Upper/inner	CRC	4-177	29.20 ± 44.74*	12
		No CRC	1-18	8.40 ± 5.58	8
	Deeper	CRC	1-37	14.67 ± 10.31	12
		No CRC	2-14	8.27 ± 3.28	8
TVA	Upper/inner	CRC	85-40691	9232.00 ± 11023.34	3914
		No CRC	1-53112	11592.47 ± 15384.84	5836
	Deeper	CRC	676-64650	18473.73 ± 20906.02	9563
		No CRC	265-44491	14430.73 ± 14044.96	8687

CRC = colorectal cancer; No CRC = no colorectal cancer; SD = standard deviation. *P < 0.05 compared to No CRC (non-parametric Mann-Whitney test).

D2-40 in adenomas in patients with CRC

MVD ranged from 4 to 177 (median 12; mean 29.20) in "hot spots" of the upper/inner parts of the adenoma and TVA ranged from 85 to 40691 μm^2 (median 3914; mean 9232.00 μm^2). MVD ranged from 1 to 37 (median 12; mean 14.67) in "hot spots" of the deeper area, and TVA ranged from 676 to 64650 μm^2 (median 9563; mean 18473.73 μm^2).

D2-40 in adenomas from patients without CRC

MVD ranged from 1 to 18 (median 8; mean 8.40) in "hot spots" of the upper/inner parts of the adenoma and TVA ranged from 1 to 53112 μm^2 (median 5836; mean 11592.47 μm^2). MVD ranged from 2 to 14 (median 8; mean 8.27) in "hot spots" of the deeper area and TVA ranged from 265 to 44491 μm^2 (median 8687; mean 14430.73 μm^2). The results for D2-40 are summarized in Table 2. A plate with illustrations of cases with lower and higher vascularization using both markers is shown in Figure 2.

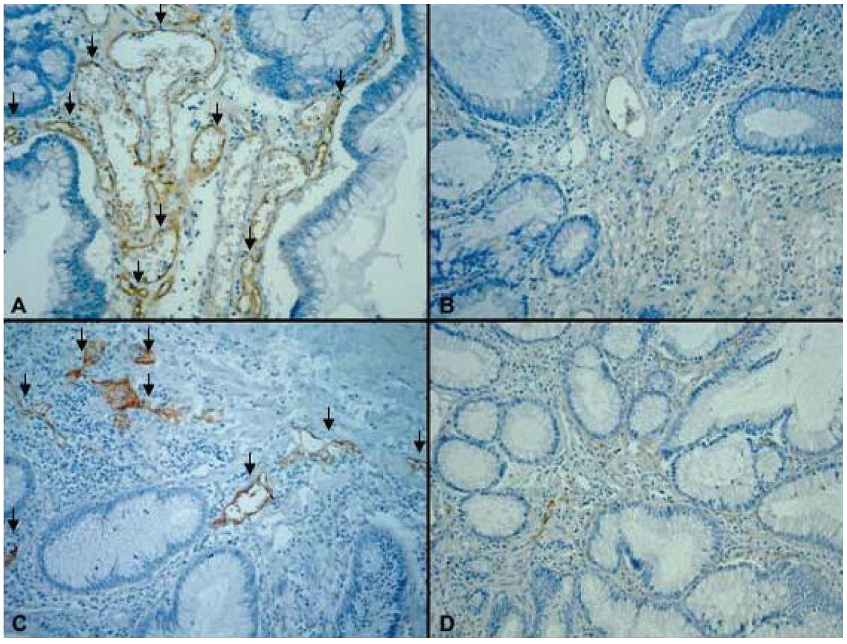

Figure 2. Various degrees of vascularization using anti-CD105 and D2-40: low and high values are shown (original magnification, 200X). *A*, CD105, High vascularization (arrows); *B*, CD105, low vascularization; *C*, D2-40, high vascularization (arrows); *D*, D2-40, low vascularization.

Statistical analysis of these data showed significantly higher values of TVA determined by immunostaining for CD105 (P = 0.019) and of MVD determined by immunostaining for D2-40 (P = 0.041) when compared with those from patients without CRC.

For both markers, there was no significant difference among histological types of adenoma (tubular, tubulovillous and villous) and MVD or TVA counts, in the groups of patients with and without CRC.

The cutoff value for TVA determined by CD105 in the upper/inner parts of the adenomas was 4386, or approximately 4400 μm^2 (sensitivity and specificity: 66.7%; predictive positive and predictive negative values: 66.7%; accuracy: 66.7%). The cutoff value of MVD determined by D2-40 in the upper/inner parts of the adenomas was 11.5 (60.0% sensitivity, 66.7% specificity, 64.3% predictive positive value, 62.5% predictive negative value, and 63.3% accuracy).

Discussion

The present data indicate a significant increase in blood microvascular area and in lymphatic microvascular counts in the upper and inner portions of adenomas removed from patients with CRC compared to those without carcinoma. Cancer cells might have an influence on the vascularization of adenomas, evidence supported by data showing increased levels of angiogenic factors in colorectal tissues distant from the primary tumor. Hanrahan et al. (26) showed that vascular endothelial growth factor (VEGF) plays a role early in tumor development at the stage of adenoma formation. Moreover, increased levels of VEGF in normal tissue collected from sites distant from the primary tumor have indicated environmental changes that could help explain our findings, although this was not directly assessed in our material.

The more significant increase in lymphatic MVD in the upper/inner areas of the adenomas is in keeping with a previous study reporting a more superficial location of lymphatic vessels in adenomas. This finding supports the hypothesis sustained by Fogt et al. (20) that superficial lymphatic vessels may be immature in normal colonic mucosa and may not communicate with deeper vessels, changing and maturing through the adenoma-carcinoma process. An equivalent assumption could be made about the increase in newly formed blood vessels detected by CD105, which suggests that they may originate superficially on adenomas, developing and meeting deeper vessels during the progression of malignancy.

The assessment of TVA using immunostaining for CD105 showed significantly higher values in adenomas from patients with CRC, while assessment of MVD did not. The opposite was seen in the assessment of lymphatic vessels using the D2-40 antibody: in contrast to MVD, TVA did not differ significantly between the two groups of lesions. These differences might reflect variations in the mechanisms of proliferation of blood and lymphatic vessels, the former affecting predominantly architectural scores, and the latter numerical scores. Unlike normal blood vessels, newly formed blood vessels incorporated during tumor angiogenesis are tortuous and dilated, a fact that could explain the higher value of TVA using CD105 in patients with CRC, an aspect supported by experimental studies (27). The higher MVD evaluated by D2-40 in patients with CRC could be explained by recent evidence showing elevated lymphatic vessel counts as an event preceding the increased number of blood vessels in early gastrointestinal tumors (28). It should be noted that computer image analysis seems to be more objective and reproducible, reducing to minimum intraobserver variability from case to case, and increasing the reliability of information in the study of angiogenesis (29).

The findings reported in the present study support the notion that neoangiogenesis and elevated lymphatic vessel counts occur in colorectal adenomas from patients with CRC, allowing us to assume that either angiogenic factors produced by the carcinoma or constitutional defects of the colorectal epithelial cells might account for these observations.

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