Assessment of angiogenesis expression and its relationship with prognosis of colorectal cancer by conventional and computer-assisted histopathological image analysis[1]

Investigação da expressão da angiogênese e sua relação com o prognóstico do câncer colorretal pela análise convencional e informatizada da imagem histopatológica

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ABSTRACT

Purpose: To quantify the degree of angiogenesis by conventional method (microvessel density, MVD) and computerized method (endothelial area, EA), and to evaluate their relationships with the prognosis of patients operated on for colorectal adenocarcinoma. **Methods**: Tumoral angiogenesis was studied by means of an immunohistochemical technique, using CD 34, on 126 patients; to quantify the angiogenesis, MVD (defined as number of microvessels per mm^2) and EA measurement (defined as the area occupied by EA in the microscope field). A computerized method, IMAGELab software was utilized to quantify endothelial area. **Results**: The mean number of microvessels was 128.6 MV/mm^2 (SD = 44.5) and the mean EA was 4.3% (SD = 2.1). The Pearson method demonstrated a low correlation coefficient between MVD and EA (r = 0.429). No relationship between MVD and EA was observed with regard to relapse-free interval and overall survival. **Conclusion**: The histological analysis of angiogenesis expression in patients with colorectal adenocarcinoma can be performed either by computer-assisted image analysis of endothelial area or by conventional microvessels counting. Both methods did not show any significant relationship between these angiogenesis parameters with relapse-free interval and overall survival.

Key words: Colonic Neoplasms. Image Processing. Computer-Assisted. Prognosis.

RESUMO

Objetivo: Quantificar a intensidade da angiogênese pelo método convencional (densidade microvasal, DMV) e pelo método informatizado, área endothelial (AE) e avaliar a sua correlação com o prognóstico de doentes operados por adenocarcinoma colorretal. **Métodos**: A angiogênese tumoral foi investigada por meio de técnica imuno-histoquímica, utilizando-se CD-34, em 126 doentes; para quantificar a angiogênese, a microdensidade vascular, definida como o número de microvasos por mm^2 e a medida da área endotelial, definida como a área ocupada pelo endotélio vascular identificada no campo microscópico foram empregadas. Um programa computadorizado, o IMAGELab foi empregado para a quantificação da área endothelial. **Resultados**: A media do número de microvasos foi de 128,6 MV/mm^2 (desvio padrão de 44,5). O método do coeficiente de Pearson demonstrou uma baixa correlação entre a DMV e a AE (r=0,429). Nenhuma correlação entre a DMV e AE com o intervalo livre de doença e tempo global de sobrevida foi observada. **Conclusão**: A análise histológica da expressão angiogênica em doentes com adenocarcinoma colorretal pode ser realizada tanto da forma informatizada, na quantificação da área endotelial, como pela convencional, na contagem dos microvasos. Ambos métodos não demonstraram relação estatisticamente significante entre estes parâmetros de angiogênese e o intervalo livre de doença e a sobrevida global.

Descritores: Neoplasias do Colo. Processamento de Imagem. Assistida por Computador. Prognóstico.

Introduction

It is known that the process of angiogenesis plays a central role in local tumor growth and in the development of distant metastases by facilitating the entry of cells into the circulation[1]. Immunohistochemical studies have demonstrated that the intensive formation of tumoral microvessels stimulated by angiogenic factors contributes towards tumor angiogenesis in colorectal cancer. New therapeutic approachs that inhibit angiogenesis process have demonstrated promising antineoplastic effects on metastatic colorectal cancer and are partially investigated in clinical trials [2]. Oxaliplatin, bevacizumab and cetuximab are some of these drugs which have been recently approved and recognized as effective in enhancing the results of chemotherapy for colon cancer. An association between the degree of angiogenesis expression and the prognosis for colorectal adenocarcinoma cases has also been reported [3, 4, 5]. A recent review on histomorphological aspects of angiogenesis and neoangiogenesis has shown that a judicious combination of microscopic angiogenic parameters should be an integral component of tumor staging system [6]. Nevertheless, it has still not become routine to assess angiogenesis, and it is possible that such difficulty might be related to the lack of an accurate method for assessing angiogenesis expression [7, 8]. The conventional way of quantifying angiogenesis consists of the counting of microvessels on a histological thin section. Lately computerized analysis of histological images have allowed the measurement of vascular parameters, and several studies have also reported a relationship between prognosis and angiogenesis expression [9, 10, 11]. The aim of the current study was to examine the relationship between angiogenesis and prognosis of colorectal cancer either by manual counting of microvessels density or by computer-assisted analysis of endothelial area.

Methods

A retrospective series of consecutive colorectal adenocarcinoma patients, who underwent surgical resection within the Departments of Gastroenterological Surgery and Pathology, at UNIFESP-Escola Paulista de Medicina, São Paulo, Brazil, was studied. The information regarding the clinical and pathological staging, surgical treatment and follow-up of the patients was obtained from the patients' medical records. The institution's Ethics Committee approved the research protocol. Patients were not included in the study if they had undergone preoperative radiotherapy or chemotherapy, if their tumors were related to familial colonic polyposis, ulcerative colitis or Crohn's disease, or if they had other tumors. The sample analyzed consisted of 126 patients (60 men and 66 women) with a mean age of 63.7 years old, ranging from 30 to 87 years. The surgical treatment was regarded as radical in 99 patients (78.6%) and palliative in 27 (21.4%). Tumors were pathologically staged according to the TNM classification, as follows: 19 patients (15.1%) as stage I; 42 (33.3%) as stage II; 36 (28.6%) as stage III; and 29 (23%) as stage IV. The tumor site was in the right colon in 33 patients (26.2%), in the left colon in 30 (23.8%) and in the rectum in 63 (50.0%) (Table 1). Patients were followed up for a mean period of 35.8 months, which allowed detection of full cancer recurrence in 23 patients (23.2%) out of the 99 patients who underwent radical surgery. There were 26 deaths, 10(38.4%) of which related to colorectal cancer.

TABLE 1 - Patients distribution with regard to mean age, gender, tumour site, TNM staging, microvessel density and endothelial area

Patients		
	Number	126
	Mean age	63.7 years
Gender		
	Male	60 (47.6%)
	Female	66 (52.4%)
Tumour site		
	Colon	63 (50,0%)
	Rectum	63 (50.0%)
TNM staging		
	I	19 (15.1%)
	II	42 (33.3%)
	III	36 (28.6%)
	IV	29 (23.0%)
Microvessel Density (MVD)		128,61 ± 44,5
Endothelial Area (EA)		4,3 ± 2,1

Immunohistochemical technique and angiogenesis analysis

New histological sections of thickness 4mm were cut from the paraffin blocks that were utilized for the histopathological study. These sections were taken from the point of greatest tumor penetration into the intestinal wall. Anti-CD34 monoclonal antibodies were utilized at a dilution of 1/250 (QD-END/10^{Ò}; Novocastra) for immunolocation of blood vessels by means of the streptavidin-biotin-peroxidase method. The angiogenesis assessment was performed via the method proposed by Weidner et al.[12]. The points of most intense vascularization (hot spots) were located by scanning the thin section under an optical microscope at a magnification of 100X, with the selection of three points. The microscope images were digitized and recorded, thereby forming an image bank of the fields selected. Images were captured using an optical microscope coupled to a color video camera and saved to disc as bmp files. Each digitized image corresponded to a microscope field with an area of 0.29 mm^2. The quantification of the tumoral angiogenesis was performed using two methods: the microvessel density (MVD), defined as the number of microvessels per mm^2, and the endothelial area (EA), defined as the percentage area occupied by endothelial cells. The counting of the microvessels was done manually on the computer monitor screen, for the three fields selected. Once each vessel

had been identified, it was marked, thereby avoiding counting it again. The number of microvessels on each thin section was determined by calculating the average for the three fields and dividing this number by 0.29, thus obtaining the number of microvessels per mm^2. Microvessels were considered to be represented by any endothelial cell or set of endothelial cells shown up by the antibodies, with clear separation from other microvessels, independent of the presence of a vascular lumen or erythrocytes[13]. For the EA assessment, the IMAGELAB software for image processing and analysis (Softium Informática Ltda., São Paulo, Brazil) was utilized [14]. From the color spectrum, it was possible to separate out the areas stained dark chestnut-brown by CD34 and then calculate the proportion of each field occupied by the stained areas (Figure 1). The results were expressed as percentages of CD34 positive pixels per recorded field. The EA score for each thin section was determined by calculating the average of the numbers obtained from reading the three points. These assessments were performed without knowledge of the clinicopathological stage and the results from the histological study using hematoxylin-eosin staining. No significant interobserver variability has been noticed.

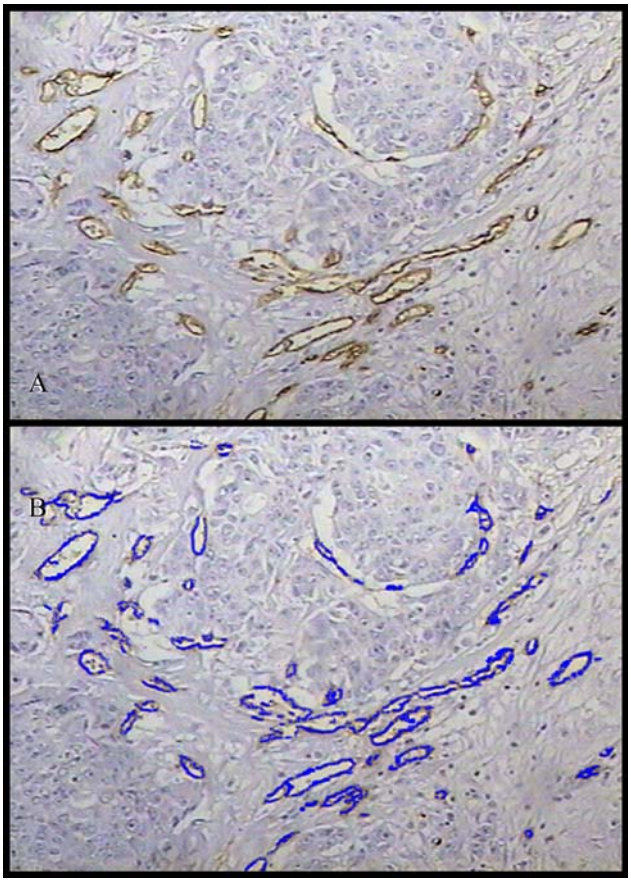

FIGURE 1 - (A) Digitized image of a "hot spot": microvessels shown up by CD34 are in dark chestnut brown. (B) Measurement of the endothelial area, using the IMAGELAB software: vascular endothelium separated by color spectrum and measured in relation to its percentage area

Statistical analysis

Data analysis were performed using statistical package SPSS software version 10.0. Cox proportional risks model test has been used to determinate which variables were significantly associated with prognostic parameters (relapse-free interval and overall survival). Gender, age, TNM staging, tumor site (colon or rectum), microvessel density (MDV) and endothelial area (EA) were analysed. To evaluate the correlation between microvessel density and the endothelial area, the Pearson linear correlation method was utilized. Differences in values were considered to be significant when $p < 0.05$.

Results

The microvessel density ranged from 56.3 to 267.8 MV/mm^2, with a mean of 128.6 ± 44.5, and the endothelial area ranged from 1.5 to 14.4%, with a mean of 4.3 ± 2.1. There was a low correlation coefficient ($r = 0.429$) between the MVD and EA measurements (Figure 2). The distribution of the demographic data are shown on the Table 1. No significant difference ($p>0,05$) was detected between age, gender, tumour site, microvessel density, endothelial area with relapse-free interval and overall survival. TNM stage was shown to be significantly associated with relapse-free interval and overall survival ($p<0,0001$) (Table 2). Evaluation via the linear correlation model of Pearson did detect positive association between MVD and EA, although a low correlation rate was noticed between them ($R=0,42$), Figure 2.

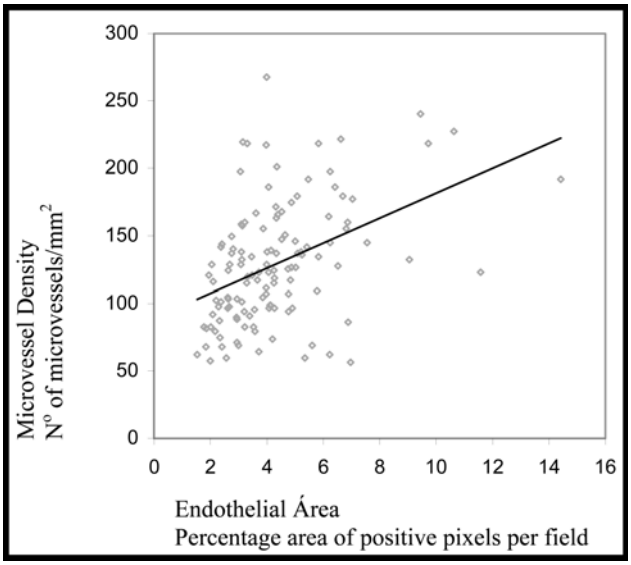

FIGURE 2 - Graph showing the correlation between MVD and EA (r=0,429)

TABLE 2 - Association of the patients variables with relapse-free interval and overall survival (Cox proportional hazard model)

	Relapse-free interval	Overall survival
Age	p=0,26	p=0,61
Gender	p=0,26	p=0,76
TNM stage	p=0,01*	p<0,0001*
Tumour site	p=0,43	p=0,20
Microvessel density	p=0,48	p = 0.67
Endothelial area	p = 0.50	p = 0.28

• statistical significant value of p.

Discussion

The measurement of microvessel density is considered to be the method with greatest validation for assessing angiogenesis [15]. In this method, the vascular endothelium is shown up by an immunohistochemical technique, and then the number of microvessels per microscope field is counted in the areas of greatest microvessel density, which are known as hot spots [12]. It has already been demonstrated that neovascularization is associated with tumor growth and that the possibility exists that tumors with greater numbers of microvessels are related to metastases [16, 17, 18]. In fact, a relationship between the degree of microvessel density and worse prognosis for colorectal cancer has been observed in several studies [4, 5, 19], although this has not been confirmed by some other authors [20, 21, 22]. These differences in results have been attributed in part to limitations in the traditional method for assessing angiogenesis [7, 8, 23]. Among other factors involved, it can be mentioned that, with the conventional method, only the number of microvessels is evaluated, while other information like the size of the vessels, for example, is disconsidered [11]. This forms the justification for the present study. Its objective was thus to determine the degree of angiogenesis in colorectal tumors, by measuring the endothelial area via a computerized method, and to study its relationship with MVD. Our first observation, as found by other authors [21, 24, 25], was that computerized readings of endothelial areas were feasible and that this would enable precise estimation of the degree of neovascularization of the tumoral tissue. However, our results demonstrated that there is a low correlation between microvessel density and the area occupied by vascular endothelium. This demonstrates the need to use new tools for quantifying tumoral angiogenesis, such as computerized analysis of histological images. Some advantages of this method are that it allows measurement of vascular parameters other than the microvessel count, such as the endothelial area [10], the vessel diameter [11] and the vascular perimeter [26], which are new parameters for assessing angiogenesis, with potential for more consistent clinical applicability. Some authors utilizing these parameters have reported on research results relating to breast tumors, with the conclusion that the greater the endothelial area is the worse the prognosis is. This would not have been found if the number of microvessels had been utilized as the measurement [10]. Analysis of the diameters, in a computerized morphometric study of tumor microvessels, has also allowed the conclusion that this parameter correlated with the development of metastases [11, 25]. In our study, neither of the two parameters studied (MVD and EA) presented any relationship with the length of disease-free survival or overall survival. However, the tumors that did not present relapse had smaller endothelial areas (4.1%) than did those that presented relapse and progression (4.5%). This difference was greater when the distant relapses were analyzed separately (5.2%). Even though these results did not show significance, this may be interpreted as a tendency, and this therefore justifies conducting studies using larger samples. Although it is well established that the growth and metastasis tumor rely on angiogenesis process, a better understanding of the biology of the tumor dissemination and molecular events is necessary[2]. The results of this investigation suggest that a more complex relationship between prognosis and angiogenesis parameters might happen. As a matter of fact some authors have reported that many tumor phenotypes and molecules (microinstability satelites, *K-ras*, p53, COX-2) regulate the VEGF expression and as a result the angiogenesis and the tumor progression [27]. Even the combination of traditional prognostic factors like TNM or Dukes colorectal cancer staging systems with molecular tumors markers have failed to better predict outcomes. In order to improve the prognostic evaluation of tumours, selection of optimal antiogenic therapy and pertinent research an association of quantitative and qualitative microscopic neoangiogenesis parameters with pathologic tumor staging system has been proposed [6]. One advantageous feature from our study was that all the images were recorded and identified in the databank, which meant that they could be audited at any time during the study, including remotely by researchers from other associated institutions. Another possible advantage of the computerized method utilized might be a lower level of observer interference, since the measurements were at least partially made by the computer. This is, however, a controversial question since there is no consensus with regard to the degree of observer interference in MVD assessments using the conventional method [8, 20, 28, 29, 30]. We were also able to observe that the measurement of EA by the computerized method was particularly useful in the evaluation of tumors with high vessel density, in which the presence of microvessels very close to each other makes manual counting difficult and laborious [9]. Since measurement of the endothelial area represents the total quantity of vascular endothelium on the histological thin section, there is no need to separately identify each vessel [10]. In our investigation the measurement of the endothelial area (EA) was made only when endothelial wall was identified by brown stain. The endothelial cell clusters, without lumen, but identified as hot spots were counted as microvessels in the evaluation of microvessel density (MDV). We regard as essential the need of a previous consistent definition of these parameters in

order to obtain an accurate expression of the measurement of the degree of angiogenesis. The greater rapidity of assessing tumoral angiogenesis provided by the computerized method for analyzing histological images may make it possible to apply this in clinical practice, considering that the slowness of the conventional method has been deemed an obstacle [9, 23]. Nevertheless, even though the present study and others have demonstrated that the assessment of angiogenesis by means of a computerized method is objective and reproducible, confirmation of this as a trustworthy method still depends on validation of the technique and definition of which parameters best relate to the prognosis. On the basis of the data from this study, we can suggest that histological analysis of angiogenesis expression in patients with colorectal adenocarcinoma can be performed either by conventional microvessels counting or by computer-assisted image analysis of endothelial area. Both methods did not show any significant relationship of these angiogenesis parameters expression with relapse-free interval and overall survival.

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