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ORIGINAL ARTICLE

Peritumoural, but not intratumoural, lymphatic vessel density and invasion correlate with colorectal carcinoma poor-outcome markers

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Abstract To evaluate whether lymphatic vessel density (LVD) and lymphatic vessel invasion (LVI) are useful markers of worse outcome in colorectal carcinoma and if LVD and LVI correlate to the classical clinical-pathological parameters, we analysed 120 cases of colorectal carcinomas selected from the files of Division of Pathology, Hospital das Clinicas, São Paulo University, Brazil. Assessment of LVD and LVI was performed by immunohistochemical detection of lymphatic vessels, using the monoclonal antibody D2-40. Higher LVD was found in the intratumoural area, when comparing with normal and peritumoural areas ($p<0.001$). However, peritumoural LVD, but not intratumoural, correlated with both colonic-wall-invasion depth ($p=0.037$) and liver metastasis ($p=0.012$). Remarkably, LVI was found associated with local invasion ($p=0.016$), nodal metastasis ($p=0.022$) and hepatic metastasis ($p<0.001$). Peritumoural LVD and LVI are directly related to histopathological variables indicative of poor outcome such as lymph-node status and liver metastasis.

Keywords D2-40 · Colorectal carcinoma · Metastasis · Lymphatic vessel density · Lymphatic invasion · Lymph-node invasion

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Introduction

Colorectal carcinoma is one of the most prevalent malignancies, being a common cause of cancer-related death, and its incidence is continuously increasing worldwide. The estimated 2006 cancer rates in Europe were 3,191,600 cancer cases diagnosed (excluding non-melanoma skin cancer) and 1,703,000 related deaths. Colorectal cancer was the second most common form of cancer (412,900, 12.9% of all cancer cases) and also the second most common cause of death from cancer (207,400 deaths or 12.2%) [6]. Since lymphatic-vessel network is recognized as the first conduit for colon-carcinoma metastasis, lymph-node invasion has been considered a marker of worse prognosis [11], as well as the number of lymph nodes examined and compromised by the carcinoma, distinguishing mesenteric tumour deposits from replaced lymph nodes. These variables should be searched together with the assessment of tumour grade, depth of wall penetration, blood-vessel invasion and tumour stage [21]. It must be acknowledged, however, that accurate identification of each criterion of this traditional checklist may not be straightforward [8]. Moreover, present assessment of these histo-

pathological features not always predicts individual outcome; therefore, additional approaches have been studied [4]. It is becoming increasingly clear that additional factors, both morphological and molecular, will be needed for future clinical management. Recent studies have also shown potential approaches for blocking the growth of lymphatic vessels to prevent tumour metastasis [1]. Blockade of vascular endothelial growth factor receptor (VEGFR)-3 pathway by specific antibodies has been reported to efficiently inhibit experimental tumour lymphangiogenesis and metastasis [24]. Moreover, recent experimental data recognized that mice lymphatic vessels have substantial plasticity during the early postnatal period, but not after that, thus suggesting that anti-lymphangiogenic therapy could possibly be safely applied to adults [9].

The search for more reproducible morphological markers and a better understanding of molecular signalling of lymphatic sprout in cancer scenario is a very motivating challenge due to the possible repercussion both in prognostic assessment and in most accurately understanding metastasis mechanisms. Recently, positive correlation between lymphatic vessel density (LVD), lymphatic vessel invasion (LVI), depth of invasion and metastases to regional lymph nodes and the liver has been reported [8]. Also, lymphatic vessel invasion was found to be related with lymph-node metastasis, and both lymphatic microvessel density and lymphatic vessel invasion were also correlated to poor outcome [13]. The preliminary findings have consistently found a positive correlation between LVD and LVI and adverse outcome [13]. However, these results are not unanimously accepted and should be cautiously considered because the degree of lymphangiogenesis alone was not recognised as an independent prognostic factor for colorectal cancer [16]. Furthermore, in spite of the potential usefulness of lymphatic microvessel density evaluations as prognostic factor, no clear-cut positive correlation has been reported between the degree of lymphangiogenesis and clinical outcome, and more data from different series are still necessary to validate these findings [20].

For this reason, herein we investigated if increased LVD as well as LVI in the primary tumour, in a large series of colon carcinomas, correlated to histopathological markers indicative of poor outcome, such as depth of tumour invasion, lymph node and liver metastasis.

Materials and methods

A series of formalin-fixed, paraffin-embedded samples from 120 colorectal carcinoma cases was retrieved from the files of the Division of Pathology, Hospital das Clinicas, São Paulo University School of Medicine (São Paulo, Brazil).

The tumours were primarily categorised according to WHO classification [7] and staged according to Tumor, Node, Metastases (TNM) classification. Clinic-pathological variables included: age, gender, macroscopic presentation, tumour size, depth of invasion, lymph-node status, TNM staging and hepatic metastasis.

D2-40 immunohistochemistry

Immunohistochemistry was carried out using the avidin–biotin–peroxidase complex assay, with the monoclonal antibody D2-40 (DAKO, Carpinteria, CA, USA) raised against an *O*-linked sialoglycoprotein. Briefly, deparaffinized and re-hydrated sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated at 98°C for 20 min; slides were, then, incubated with 0.3% hydrogen peroxide in methanol for 30 min, followed by incubation with Normal Horse Serum (Vector Laboratories, CA, USA) for 20 min, at room temperature, before incubating with the primary antibody diluted 1:100, overnight at 4°C. Sections were then sequentially washed in phosphate-buffered saline 1× and incubated with Biotinylated Universal Secondary antibody (Vector Laboratories) for 30 min, Vectastain® Elite ABC reagent (Vector Laboratories) for 45 min at 37°C, developed with 3,3′-diamino-benzidine (DAKO) for 10 min and counterstained with hematoxylin–eosin. Negative controls were performed by omitting the primary antibody and, as positive control, tonsil tissue was used.

Immunohistochemical evaluation

Immunohistochemical reaction of D2-40 was evaluated considering its expression in the membrane/cytoplasm of endothelial cells. Evaluation was performed blindly, and LVD was assessed as described by Weidner et al [23] with slight modification. For LVD assessment, microvessel was defined as a single endothelial cell or a cluster of endothelial cells positive for D2-40, sitting around a visible lumen clearly separate from adjacent microvessels and from other connective-tissue components. Additionally, as lymphatic vessels could generally appear as distorted and overlapped structures in cancer setting, the packed vessels were assumed as one lymphatic unit. The number of vessels was quantified at ×200 magnification. The median of microvessels counted in ten hot-spot fields was defined as LVD. Each hot spot corresponds to a number of vessels confined to an area of 0.15 mm^2. Additionally, a cut-point of 4 micro-vessel invasion (median LVI value, evaluated in the same areas where LVD was analysed) was used for comparison with pathological variables. Both LVD and LVI were evaluated using D2-40 immunostained lymphatic vessels.

Statistical analysis

Data were stored and analyzed using the SPSS statistical software (for Windows, version 14.0, Chicago, IL, USA). The Shapiro–Wilk test was applied to assess normality of the results. Data was examined for statistical significance using the Student's *t*, the one-way analysis of variance (ANOVA), the Mann–Whitney *U*, the Kruskal–Wallis and the Pearson's chi-square (χ^2) tests, as appropriate, being threshold for significance *p* values <0.05.

Results

We have studied 120 colon carcinoma samples from patients with a median age of 64, ranging from 24 to 95 years, which included 53 women and 67 men.

The median value of LVD, assessed by D2-40 immunoexpression, in intratumoural areas was significantly higher than those in normal stroma ($p<0.001$, Fig. 1). In addition, intratumoural LVD counting exhibited about twofold more lymphatics than at the periphery of the tumour ($p<0.001$, Table 1).

Table 2 depicts the correlation between the clinical-pathological variables and LVD (peri- and intratumoural). No association was found between age or gender of patients and tumoural LVD (data not shown). Peritumoural LVD was associated with depth of wall invasion ($p=0.037$), hepatic metastasis ($p=0.012$) and TNM staging ($p=0.044$). We found significant differences ($p<0.001$) between the evaluation of lymphatic invasion with the specific lymphatic marker D2-40 and haematoxylin–eosin stain: 74.2% (89 of 120) against 48.3% (58 of 120), respectively.

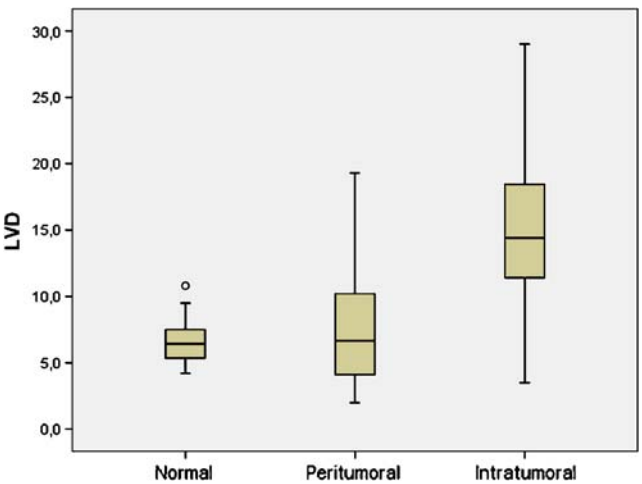

Fig. 1 Representation of lymphatic vessel density among the different colorectal areas

Table 1 Comparison of lymphatic vessel density (LVD) among the different tissue regions using the Student's *t* test

Tissue regions	LVD (median)	*p*
Normal vs peritumoural	6.4 vs 6.8	0.682
Normal vs intratumoural	6.4 vs 14.6	<0.001
Peritumoural vs intratumoural	6.8 vs 14.6	<0.001

The median values represent the number of lymphatic vessels counted in hot-spot areas.

Table 3 shows the correlation between invasion, presented both as positive and as number of invaded vessels (above the median value), and the histopathological variables. As for vessel density, no correlations were found between lymphatic invasion and age of diagnosis or gender (data not shown). Lymphatic invasion (Fig. 2) was significantly associated with presence of nodal metastasis ($p=0.022$), and a trend for cases with hepatic metastasis to present lymphatic invasion ($p=0.064$) was also observed. When considering the number of invaded lymphatic vessels (>4), we found a significant correlation with depth of invasion ($p=0.018$), the presence of both nodal and hepatic

Table 2 Associations of LVD with the histopathological data

Histopathological variables	*n*	Peritumoural LVD[a]		Intratumoural LVD[b]	
		Median	*P*	Mean	*p*
Tumour size			0.116		0.817
≤5	65	6.1		15.0	
>5	54	7.1		15.2	
Macroscopic type			0.574		0.792
Exofitic	24	6.4		15.6	
Ulcerative	45	6.0		14.5	
Infiltrative	12	8.9		14.5	
Sessile-ulcerated	39	6.8		15.6	
Depth of invasion			0.037		0.551
T1 + T2	22	5.4		14.4	
T3 + T4	98	7.2		15.2	
Nodal metastasis			0.414		0.660
Absent	60	6.8		14.8	
Present	60	6.4		15.3	
TNM			0.044		0.937
I	16	4.8		14.6	
II	33	7.1		14.9	
III	36	5.6		15.0	
IV	35	9.1		15.6	
Hepatic metastasis			0.012		0.559
Absent	85	5.8		14.9	
Present	35	9.1		15.6	

[a] Mann–Whitney *U* test and Kruskal–Wallis test were used when sample-grouping >2 (data do not follow a normal distribution)
[b] Student's *t* test and one-way ANOVA were used when sample-grouping >2 (data follow a normal distribution)

Table 3 Relationship between lymphatic invasion and clinical-pathological parameters using the Pearson's chi-square (χ^2) test

	n	Positive invasion		Invasion >4	
		%	*p*	%	*p*
Tumour size			0.696		0.790
≤5	65	75.4		29.2	
>5	54	72.2		31.5	
Macroscopic type			0.128		0.943
Exofitic	24	66.7		25.0	
Ulcerative	45	68.9		31.1	
Infiltrative	12	100.0		33.3	
Sessile-ulcerated	39	76.9		30.8	
Depth of invasion			0.212		0.018
T1 + T2	22	63.6		9.1	
T3 + T4	98	76.5		34.7	
Nodal metastasis			0.022		0.046
Absent	60	65.0		21.7	
Present	60	83.3		38.3	
TNM			0.107		0.009
I	16	68.8		12.5	
II	33	60.6		21.2	
III	36	77.8		25.0	
IV	35	85.7		51.4	
Hepatic metastasis			0.064		0.001
Absent	85	69.4		21.2	
Present	35	85.7		51.4	

metastasis (p=0.046 and p=0.001, respectively) and TNM staging (p=0.009).

Discussion

In the present study, we found a significant difference between LVD in the normal mucosa and intratumoural stroma (p<0.001). The results herein reported strongly correlated both peritumoural LVD and peritumoural LVI with the presence of hepatic metastasis. This association was already described within tumoural areas and reinforces the importance of our results. Recently, Parr and Jiang [18] elegantly described lymph-vessel expression by using real-time quantitative polymerase chain reaction assay for several lymphangiogenic markers and found that VEGFR-3, Prox-1, podoplanin and 5′-nucleotidase were higher expressed in colorectal carcinoma in comparison to the normal mucosa. Indeed, we found similar LVD in normal and peritumoural area but higher LVD in the intratumoural area (p<0.001). This is interesting because one can hypothesise that intratumoural area is more liable to receive lymphangiogenic stimulation during the carcinogenic development [15]. In spite of that, higher LVD does not necessarily mean that these conduits are more prone to facilitate metastatic spread; on the contrary, our results strengthened that intratumoural lymphatics in fact are not adequate route for malignant-cell escape and that functional lymphatics in the tumour margin are sufficient for lymphatic metastasis [17]. Recently, Kuroyama et al. [10] found higher intratumoural lymphatic density in cases with lymph-node metastasis than in those without metastasis but, as we found in our study, they did not observe correlation with tumour size, depth of tumour invasion, distant metastasis or TNM stage. Interestingly, we did not find significant correlations between both peritumoural and intratumoural LVD and lymph-node metastasis, even considering that we found a higher intratumoural LVD.

The number of lymph nodes evaluated after surgical resection is positively associated with survival of patients with stage II and stage III colon cancer [3]. Saad and collaborators [19] found that high LVD correlates with both depth of invasion and lymph-node metastasis. We found a significant association between peritumoural LVD and depth of invasion (p<0.037) and between the number of lymphatic vessels invaded by the tumour and depth of invasion (p<0.018). Liang et al. [11] emphasised that lymphatic microvessel density is important in colorectal carcinoma to predict lymph-node metastases mainly associated to lymphatic vessels' diameter (not assessed in the present study).

Importantly, we observed a strong correlation between peritumoural LVD and lymphatic invasion (≥4 lymphatic vessels invaded; p=0.0001) and hepatic metastasis (p=0.012). It is well known that, at the time of diagnosis of colorectal cancer, 20% of the patients already have liver metastasis, and 30% of the patients will develop metastasis afterwards [14, 19]. These findings could be decisive for future investigations regarding the surgical resection options of the colorectal hepatic metastasis [3]. Furthermore, tumour cells metastasized to the liver have certainly

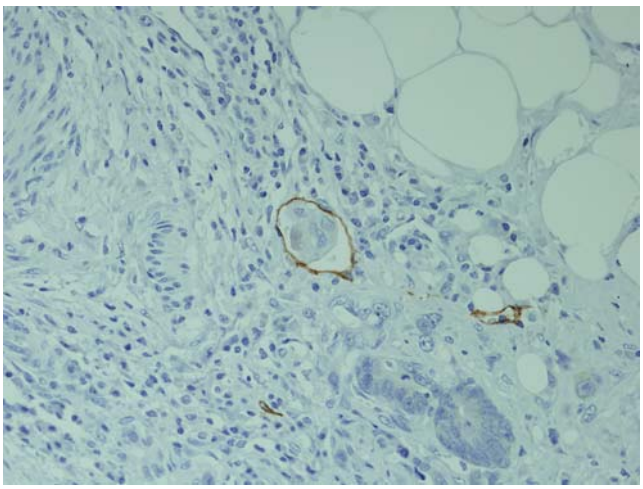

Fig. 2 Peritumoural lymphatic vessel invasion (D2-40 staining, ×40 magnification)

crossed the hepatic sinusoidal endothelial barrier and completed steps of metastatic cascade [2]. Accordingly, further studies exploring the earlier stages of colorectal metastasis, proliferation and new vessel formation as well as mechanisms to disturb cell survival are needed [5]. The more exciting results were observed with lymphatic invasion highlighted by D2-40, which positively correlated with the lymph-node status and liver metastasis. Cases with more than four lymphatic vessels invaded showed significantly more hepatic metastasis. Indeed, the correlation we observed between lymphatic invasion and TNM staging ($p=0.009$) also corroborates the above-mentioned findings. In contrast to these findings, some recent data indicate that extensive lymphangiogenesis indeed occurs in colorectal carcinoma, but there are controversial data concerning the value of the degree of lymphangiogenesis as an independent prognostic factor [13, 16]. The significant differences found between lymphatic invasion evaluated with haematoxylin–eosin stain and with the lymphatic marker D2-40 ($p<0.001$) emphasises the importance of the use of a specific marker to identify lymphatics. As compared with blood capillaries, lymphatic vessels, principally those within the tumour mass, have poorly developed junctions with frequently large inter-endothelial gaps, with discontinuous or completely absent basement membranes. The recent discovery of specific lymphatic vessel markers and their corresponding antibodies have aided the identification of lymphatic vessels. Indeed, D2-40 importantly enhances the lymphatic endothelial cells borders, highlighting the presence of lymphatic invasion in the tumours [19].

Colorectal carcinoma is one of the most prevalent cancers worldwide, and lymphatic metastatic route was long considered as an important parameter of worse outcome [8]. Due to the recent development of finer immunohistochemical markers, lymphangiogenesis was rediscovered in oncologic pathology enabling more accurate counting of LVD in all types of neoplasms, with special emphasis on carcinomas [12]. The results herein presented, using the specific lymphatic marker D2-40, indicate that peritumoural LVD and LVI are reliable parameters to predict poor prognosis and contribute to identify patients more prone to develop hepatic metastasis.

Conflict of interest statement We declare that we have no conflict of interest.

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