Role of lymphatic vessel density in colorectal cancer : prognostic significance and clinicopathologic correlations

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Backgrounds and aims : **Over the past decades the identification of several molecules that are expressed specifically in the lymphatic endothelial cells has resulted in marked advances in the field of lymphangiogenesis. We aimed to measure LVD in colorectal cancer patients and to compare it with microvascular density (MVD) – a marker of angiogenesis – and patients' clinicopathological parameters and survival, as the measurement of lymphatic vessel density (LVD) has been documented in various tumor types, including colorectal cancer.**

Patients and methods : **Fifty one patients who had undergone surgical resection for stage I-III colorectal cancer entered this study. LVD and MVD were determined immunohistochemically with the use of D2-40 and CD34 antibody respectively. The evaluation of LVD was performed by both visual and computer-aided image analysis.**

Results : **The majority of lymphatic vessels were located in the peritumoral areas rather than within the tumor. The results obtained from the image analyzer correlated significantly with the data obtained using visual counting with light microscopy. Both visual and image analysis LVD failed to correlate with patients' age and gender and tumor location, stage, grade, MVD count and survival.**

Conclusions : **The biologic role of the lymphatic vasculature in tumor progression remains controversial. The present study failed to associate LVD with outcome markers and prognosis and further studies would be required to verify our results.** (Acta gastroenterol. belg., **2015**, 78, **223-227**).

Key words : colorectal cancer, lymphatic vessel density, microvascular density, and lymphangiogenesis.

Introduction

The growth of tumors to larger than a few millimeters is dependent on the development of new blood vessels, in order to support their metabolic requirements (1,2). The sprouting of new blood vessels from pre-existing capillaries, a process also known as angiogenesis, consists of several steps, including the proliferation and migration of endothelial cells into the tumor, and the eventual formation of new capillary tubes (3). The newly formed tumor vessels are chaotic with widened interendothelial junctions and discontinuous basement membrane, features that enhance tumor cell entry into the circulation and hence distant metastasis (4,5).

Besides blood vessels the metastatic spread of cancer cells can occur through lymphatic vessels via pre-existing and/or via newly formed ones (6,7). In contrast to angiogenesis, the study of the lymphatic system has remained relatively neglected mainly due to the absence of known lymphatic endothelium-specific markers. Over the past decades the identification of several molecules that are expressed specifically in the lymphatic endothelial cells has resulted in marked advances in the field of lymphangiogenesis (8,9). The measurement of lymphatic vessel density (LVD) has been documented in various tumor types and has also been related to prognosis and early metastasis (10-12). Among the several released markers the D2-40 antibody has shown staining reaction in lymphatic channel endothelium, but not in the adjacent capillary (13). This antibody reacts with an O-linked sialoglycoprotein (MW 40K) found on lymphatic endothelium, fetal testis and on the surface of testicular germ-cell tumors (14,15).

The aim of our study is to measure LVD with the use of D2-40 antibody in colorectal cancer patients and to compare it with microvascular density (MVD)-a surrogate marker of angiogenesis- and patients' clinicopathological parameters and survival.

Materials and methods

A total of 51 colorectal cancer patients who were treated at our surgical department between 2005 and 2006 were included in this study. All patients underwent a potentially curative resection, defined as the removal of all of the macroscopic cancer tissue with absence of microscopic residual tumor. None of the patients had received chemotherapy or radiotherapy prior to surgery or died in the peri- and post-operative period (within 45 days after surgery). Patient data (gender and age) and tumor characteristics including, location, grade of differentiation and stage were ascertained through patient surgical and pathologic records. The tumors were staged according to the TNM stage. The median duration of follow-up was 60 months (range, 35-64 months) and included information regarding disease-free and overall

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Submission date : 19/09/2014
Acceptance date : 03/03/2015

survival. The study protocol was approved by the hospital's Research Ethics committee and all patients provided informed consent before study enrolment.

Formalin-fixed, paraffin-embedded tissue sections, 4 μm thick, were deparaffinised with xylene, dehydrated in ethanol and incubated with 3 % hydrogen peroxidase. Antigen retrieval was performed by heating slides in citrate buffer (10 mM, pH 6.0) at 95°C for 30 minutes. The primary monoclonal antibodies used were CD34 (Dako, 1 :50) and D2-40 (Dako, 1 :50). All sections were incubated overnight with the primary antibody at 4°C. The sections were then treated with peroxidase using the labeled polymer method with DAKO EnVision+ Peroxidase (Dako Corp., Carpinteria, CA) for 30 minutes. The peroxidase reaction was visualized with liquid 3, 3'-diaminobenzine substrate kit (Zymed Laboratories). Sections were then counterstained in hematoxylin. Negative control sections were stained by omitting the primary antibody.

Results were interpreted by two independent pathologists blinded to each other's findings and to the patients' data. Slides were examined under a light microscope at low magnification and the areas with the greatest density of lymphatic and blood vessels were selected (hot spots). Microvessel counting was performed in five fields of the hot spots at 400× magnification and the mean value was used for further analysis. The measurement of lymphatic vessel density, on D2-40-immunostained sections, was performed by both visual and computer-aided image analysis methods. Computerized image analysis was performed using a semi-automated system with the following hardware features : Intel Pentium Dual-Core, Digital Camera Sony Cyber-shot (5 m digital analysis), Microscope Olympus CX-31 and the following software : Windows XP/NIS-elements image analysis software, Nikon 2009. Any brown-stained endothelial cell, individually or in cluster, that was clearly separate from adjacent microvessels, tumor cells or other connective tissue elements was considered as a single countable microvessel. There was no restriction on the size of a countable microvessel. Vessels with muscular walls were not counted. Vessel lumen and red cells were not used to define a microvessel. Areas with a dense leukocytic or hemorrhagic infiltration were excluded from the analysis.

A standard statistical software package SPSS (SPSS Inc, Chicago IL) was used in the analysis. Descriptive statistics were calculated for all variables. Continuous variables were normally distributed and are presented as mean ± SD and categorical variables are presented as percentages. The Pearson correlation was used to measure the association between continuous variables. Categorical variables were analyzed with the chi-square test or Fisher's exact test as appropriate. Means were compared using the t test or one-way ANOVA where appropriate. LVD was treated as a continuous variable but also as a categorical, divided in two groups by the mean value. Disease-free and overall survival was estimated by the Kaplan-Meier method and compared with the log rank test. P values less than 0.05 were considered statistically significant.

Table 1. — **Patient and tumor characteristics**

Age (mean ± SD)	70.9 ± 9.3
Gender, number (%)	
Male	18 (35.3%)
Female	33 (64.7%)
Tumor Location, number (%)	
Colon	22 (43.1%)
Rectal	29 (56.9%)
TNM Stage, number (%)	
I	14 (27.5%)
IIA	19 (37.3%)
IIIA	3 (5.9%)
IIIB	10 (19.6%)
IIIC	5 (9.8%)
Grade of Differention, number (%)	
Well	4 (7.8%)
Moderate	43 (84.3%)
Poor	4 (7.8%)
LVD (mean ± SD)	
Visual	4.5 ± 3.2
Image analysis	4.8 ± 3.0

Results

The patients' ages ranged from 42 to 91 years ; 18 were male and 33 were female. The majority of patients had stage IIA (37.3%) and grade II (84.3%) tumors. Patient and tumor characteristics are summarized in Table 1.

Using the visual counting method we found a mean LVD value of 4.5 ± 3.2 and 39.6% of the enrolled subjects had LVD values above this level (Fig. 1). With image analysis we found a mean LVD value of 4.8 ± 3.0 and 62.7% of the patients had values above this level. The results obtained from the image analyzer correlated significantly with the data obtained using visual counting with light microscopy (Fig. 2). The majority of lymphatic vessels were located in the peritumoral areas rather than within the tumor. Intratumoral lymphatic vessels were extremely rare and thus not included in the study analysis.

The mean value of MVD was significantly higher than that of LVD (7.4 ± 2.2 vs. 4.5 ± 3.2 ; $p < 0.001$), no significant correlation was found between the two markers (Table 2). Both visual and image analysis LVD failed to correlate with patients' age and gender and tumor location, stage and grade. No significant association was found with lymph node status (Table 2, 3).

Kaplan-Meier analysis showed no association between visual and image analysis LVD and overall survival (Fig. 3). No correlation was found between visual LVD and disease-free survival. Determination of LVD with image analysis showed a trend towards shorter time to recurrence in patients with low LVD values, compared to

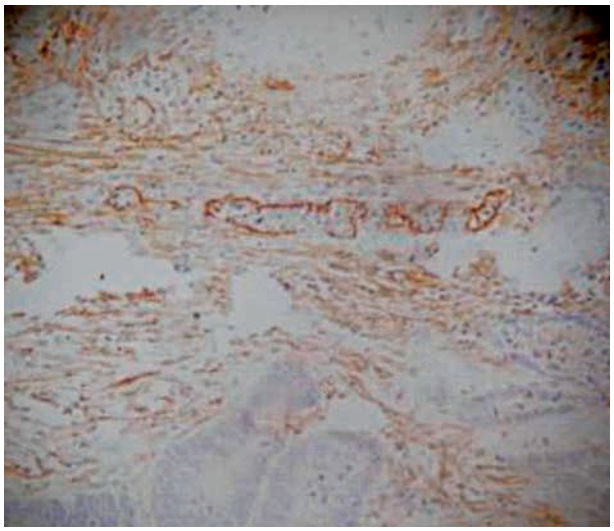

Fig. 1. — Immunohistochemical stained lymphatic vessels with D2-40 antibody.

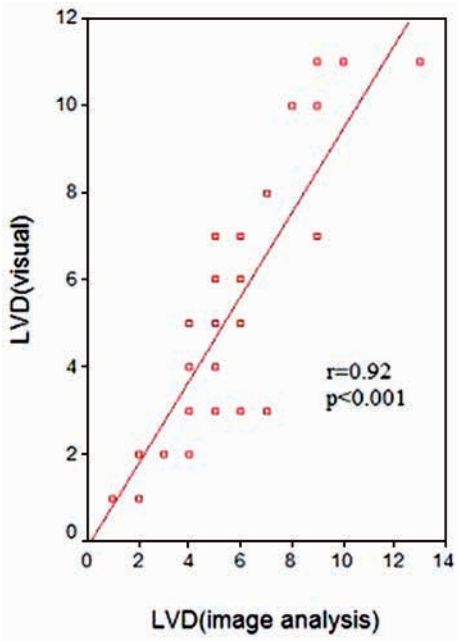

Fig. 2. — The scatter plot reveals a significant correlation between LVD values obtained with the optical method and those determined through image analysis.

those with high, yet this association was not statistically significant (log-rank test ; p = 0.08).

Discussion

Tumor metastasis to lymph nodes is a crucial step in the progression of cancer. Regional lymph nodes are the first sites to develop metastases, either draining via pre-existing afferent lymphatic vessels and/or via newly formed lymphatic capillaries. The identification of molecular markers that discriminate between lymphatic endothelium and blood-vessel led to the development of the field of lymphangiogenesis research (8,9). The present study focused on the measurement of LVD in colorectal cancer and showed no association with patients' clinicopathological parameters and prognosis.

In agreement with our results a study by Duff *et al*., in 30 colorectal cancer specimens, failed to show a correlation between LYVE-1 stained LVD and any clinicopathological variable. The lymphatic vessels were mainly located in the peritumoral area and were significantly lower than the MVD count (stained with CD34) ; in contrast to our data LVD was correlated with MVD (16). A study by Liang *et al*., in 87 patients with T1 colorectal cancer, reported that intratumoral podoplanin stained lymphatic vessels were extremely rare and that the evaluation of the diameter and density of lymphatic microvessels in the peritumoral area but not LVD alone were independent factors associated with lymph node metastases (17). On the other hand a study by Matsumoto *et al*., consisted of 106 colorectal cancer patients, although failed to show a correlation between podoplanin stained LVD and lymph node involvement reported that high intratumoral LVD was associated with depth of invasion, presence of distant metastasis, clinical stage and was an independent prognostic factor for overall survival (18). In the same manner a study in 90 colorectal cancer patients by Saad *et al*., showed that intratumoral D2-40 stained LVD correlated significantly with lymphovascular invasion, lymph node, and liver metastases independent of tumor stage. The LVD was not significantly different from the MVD count (identified by CD31) ; there was a significant correlation between the two markers (19). A study by Filho et al of 120 colorectal carcinoma cases, found that D2-40 stained LVD count was higher in the intratumoral area compared to the peritumoral, however only peritumoral LVD correlated with stage, depth of invasion and liver metastasis. No association was found with nodal metastasis (20).

Table 2. — **Bivariate correlations between LVD and age, number of involved lymph nodes and MVD**

	LVD (visual)		LVD (image analysis)	
	r	P-value	r	P-value
Age	0.08	0.57	0.08	0.57
Number of affected lymph nodes	-0.01	0.92	-0.08	0.57
MVD	-0.03	0.83	-0.01	0.92

Table 3. — **LVD correlation with clinicopathological parameters**

Characteristics	LVD (visual)	P-value	LVD (image analysis)	P-value
Gender		1.00		0.62
Male	4.5 ± 3.1		5.1 ± 3.1	
Female	4.5 ± 3.2		4.7 ± 3.0	
Tumor Location		0.89		0.66
Colon	4.6 ± 3.4		5.0 ± 3.6	
Sigmoid-Rectal	4.4 ± 3.1		4.7 ± 2.5	
TNM Stage		0.78		0.72
I	5.0 ± 3.2		5.5 ± 3.5	
IIA	4.5 ± 3.6		4.8 ± 3.3	
IIIA	2.3 ± 2.5		2.7 ± 2.3	
IIIB	4.8 ± 2.8		4.8 ± 2.0	
IIIC	4.2 ± 2.9		4.6 ± 2.7	
T		0.36		0.73
T1	7.0 ± 1.4		6.5 ± 1.0	
T2	3.8 ± 3.0		4.7 ± 3.5	
T3	4.7 ± 3.3		4.8 ± 2.9	
N		0.86		0.70
N0	4.7 ± 3.4		5.1 ± 3.4	
N1	4.2 ± 2.9		4.3 ± 2.2	
N2	4.2 ± 2.9		4.6 ± 2.7	
Grade of Differention		0.23		0.20
Well	2.8 ± 1.0		3.5 ± 1.3	
Moderate	4.9 ± 3.3		5.2 ± 3.1	
Poor	2.8 ± 2.5		2.8 ± 2.2	

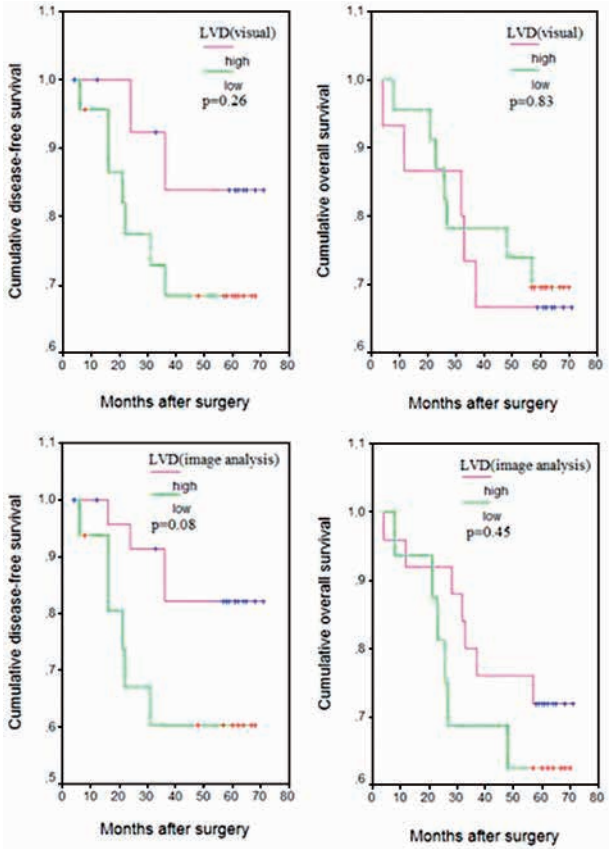

Fig. 3. — Kaplan Meier disease-free and overall survival curves with regard to LVD expression.

The contradicting results about the role of LVD in tumor progression may be due to differences in methodology such as antibody selection, counting protocols and selection of the field in which to count. In an attempt to overcome the inherent subjectivity of visual LVD counting the present study used computer aided image analysis. D2-40 microvessel densities determined by direct microscopy and image analysis were significantly correlated and provided overall with the same results. Although no ideal lymphatic endothelial marker exists, the monoclonal D2-40 antibody-used in this study- has been presented as one of the most reliable markers with a high specificity and sensitivity for lymphatic vessels (21). However, it must be stated that the use of single staining techniques with lymphatic specific markers may be unable to distinguish newly proliferating lymphatic vessels from pre-existing ones. In view of this notion it has been proposed that the method most likely to reflect ongoing lymphangiogenesis would be the analysis of proliferating lymphatic endothelial cells. Nonetheless cell proliferation is a dynamic process which may not be fully depicted by immunohistochemistry, which is a static procedure involving the evaluation of tissue sections at specific time points in tumor progression (17,21).

The presence of a high density lymphatic vasculature, although important, remains only a step in the multifactorial process of metastases from primary tumor to lymph nodes. This is illustrated by the lack of consistency

between studies regarding the impact of LVD count on lymph node status and prognosis (16-20). The present study failed to show a correlation between LVD and patients' disease-free and overall survival. Besides the presence of methodological variation with other published studies, this observation may also reflect the ability of colorectal tumors to rely more on pre-existing lymphatic vessels than on newly formed ones.

Another issue of debate among studies investigating lymphagiogenesis is the functional and prognostic significance of intratumoral vs. peritumoral lymphatics. While some studies have documented the presence of intratumoral lymphatics and provided with evidence of a direct correlation with nodal status and survival, others not only have failed to provide with such results but also showed that peritumoral lymaphatics is the predominant form found in tissue sections and is associated with poor outcome markers (16-20). In the present study LVD was mainly located in the peritumoral area. This finding is in agreement with previous observations showing compression and lack of function of intratumoral lymphatic vessels due to increased interstitial pressure, generated by the proliferating tumor cells (22,23).

The present study failed to show an association between angiogenesis and lymphangiogenesis. The number of blood vessels was significantly higher than that of lymphatic vessels an observation probably reflecting a more prominent role of the former in tumor biology. Although the pathways regarding the formation of new lymphatics and blood vessels may share some common stimulants, the lack of correlation between LVD and MVD implies the presence of different underlying mechanisms (24,25).

Although our knowledge in the biology of lymphagiogenesis has expanded over the past decades, the clinical role of the lymphatic vasculature in tumor progression remains controversial. This discrepancy may reflect differences in methodology and/or the presence of lymphagiogenic-independent pathways, such as recruitment of pre-existing vessels. Using a specific lymphatic marker and computer aided image analysis, the present study failed to associate LVD with outcome markers and prognosis. Further larger-scale studies are required to verify our results.

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