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Microvascular density in non-Hodgkin B-cell lymphomas measured using digital morphometry

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Abstract

Introduction: The growth of solid tumors requires the development of microvessels, therefore tumor expansion depends on angiogenesis. Microvessels provide nutrients and oxygen and remove catabolytic substances, while endothelial cells produce growth factors for tumor cells in a paracrine fashion. The microvascular component of a tumor also plays a role in the metastatic capacity of the tumor, enabling the tumor cells to spread to distant locations by providing a large endothelial surface. *Aim*: The purpose of this study was to review the literature about angiogenesis regarding malignant lymphomas and to perform basic measurements by means of digital morphometric methods in large B-cell lymphomas and follicular lymphomas. *Materials and Methods*: After thorough analyzing currently available assessment methods, we performed angiogenesis assessment on 19 randomly selected cases, from paraffin-embedded specimens using digital morphometry. We used immunohistochemistry and the CD34 antigen to mark microvessels. We measured average vascular diameter and a previously successfully applied digital morphometric method to quantify the extent of endothelial area. *Results*: According to literature data, our knowledge and understanding of angiogenesis grew rapidly from early studies such as Folkman's classic paper. Many studies showed that angiogenesis plays a key role in the biology of tumors and therefore the study of angiogenesis might open new therapeutic possibilities. There have been many studies of angiogenesis in malignant lymphomas, however not as many articles as in other tumor types. Our morphometric studies showed there are statistically significant differences between diffuse large cell lymphoma (DLBCL) and follicular lymphoma (FL) regarding average vascular diameter and that high grade lymphomas tend to have a greater CD34+ endothelial area.

Keywords: angiogenesis, malignant lymphomas, digital morphometry.

Introduction

The growth of solid tumors requires the development of microvessels, therefore tumor expansion correlates with the extent of angiogenesis [1]. Microvessels provide nutrients and oxygen and remove catabolytic substances, while endothelial cells produce growth factors for tumor cells in a paracrine fashion [2]. A well-developed microvascular system also facilitates local expansion of the tumor since endothelial cells secrete cellular matrix-degrading enzymes [3]. The microvascular component of a tumor also plays a role in the metastatic capacity of the tumor, enabling the tumor cells to spread to distant locations by providing a large endothelial surface that increase the opportunity of the cancerous cells to enter the circulation. Once recognized, the sequential nature of tumor vascularisation has been extensively studied. The histological quantification of human tumor angiogenesis was introduced by Weidner N *et al.* in 1991 [4]. He and his colleagues used immunohistochemical techniques to highlight tumor blood vessels with antibodies to FVIII-related antigen. Since then other markers have been employed, including CD31, CD34 and CD105, among others [5, 6]. Highlighting the vascular wall made it possible to numerically count microvessels, announcing the emerging of a new parameter that might be used in the characterization of solid tumors.

Ever since, the quantification of tumor angiogenesis in order to predict tumor behavior, has always been in the attention of pathologists. In this regard, the works of Folkman J *et al.* can be considered very important in establishing those parameters that are usually need to be measured in order to describe angiogenesis [1]. These parameters include: vessel number, endothelial cell hyperplasia and cytology, introducing for the first time the term intratumoral microvascular density (MVD). The reason why these parameters should be studied is to yield important information on the relationship to other clinicopathological tumor characteristics and help testing of antiangiogenic therapies.

Aim

Although many evidence suggests that increased MVD in many carcinoma types correlates with the increased risk of metastatic spread and subsequently a poor prognosis, there is much less information available regarding the significance of MVD in malignant lymphomas. There are relatively few quantitative results published [7, 8]. The interpretation of the results in

many cases is made difficult by the various study methods and lymphoma terminology. Our aim was to measure angiogenesis in selected cases of B-cell lymphomas, using anti-CD34 as an endothelial cell marker and a semi-automated image analysis. We included in the study one normal lymph node.

Materials and Methods

The study material consisted of surgical biopsies of lymph nodes from 19 patients with suspected diagnosis of neoplastic disease. Specimens included: 12 cases of diffuse large cell lymphoma (DLBCL), seven cases of follicular lymphoma (FL), with nodular (FLn) and diffuse (FLd) growth pattern and one normal lymph node.

Immunohistochemistry

Immunohistochemical study was performed on paraffin-embedded tissues using EnVision Plus detection system (Dako, Denmark) and monoclonal (mouse) primary antigen against CD34 (Class II, clone QBEnd 10, Dako, Denmark) to highlight vascularity. Heat-induced antigen retrieval was utilized with a microwave oven; the staining process was achieved using a Dako Immunostainer. Other markers used for primary diagnosis: CD3 (clone MS401, LabVision, USA), CD20 (clone L26, Dako, Denmark), CD5 (clone 54F6, Dako, Denmark) and Ki-67 (Dako, Denmark). DAB (diamino-benzidine) was used as chromogen, counterstained with Hematoxylin dye. Normal vasculature of interfollicular areas was considered as positive control, in the lymph node without any lesion.

Digital morphometry

From each case, ten randomly chosen microscopic fields were captured (Figure 1). We defined the CD34+/ tumor area ratio as the ratio of microvessels per tissue unit area (Figure 2). We used a Nikon Coolscope digital image acquisition system for image capturing.

We measured the following parameters:

(a) average diameter of vascular structures (ob. 20×) [VascDiam];

(b) endothelial area (CD34+)/tumor area ratio (ob. 4×) [EA/tumor].

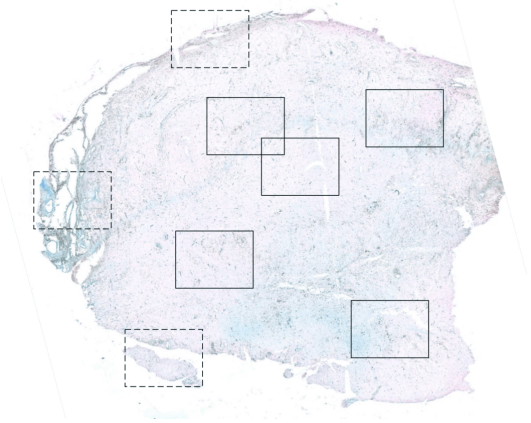

Figure 1 – ***The method of choosing the optimal field for measuring extent of microvascularity (continuous line – correct spot, dashed line – incorrect spot).***

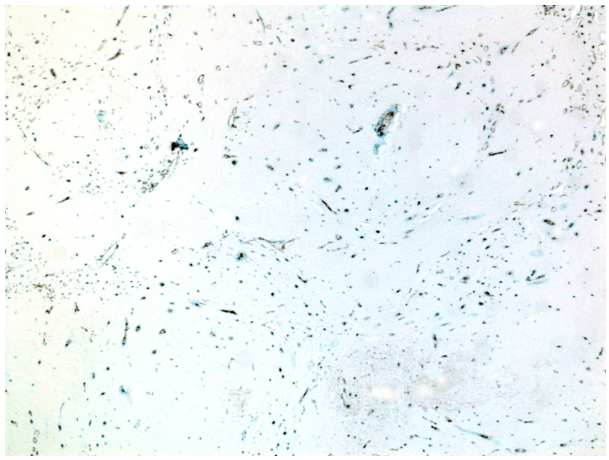

Figure 2 – ***Color image of a typical field, that was later used for microvascular density analysis.***

For the digital analysis, ImageJ software (Rasband W, National Institutes of Health, USA) [18] and a macro code were used. This method differs from other similar digital morphometry methods in that it can be tailored to specific needs, using HSB (hue, saturation, brightness) color filtering [14]. The measurement of vascular diameters was performed using Lucia software and its "Measure area" function.

The morphometric analysis that was aimed to measure the EA/tumor area ratio consisted of two main phases. The *first phase* is the colorimetric segmentation, which results in a color image (Figure 3) that contains only the desired tissue structures (in our case the CD34+ areas).

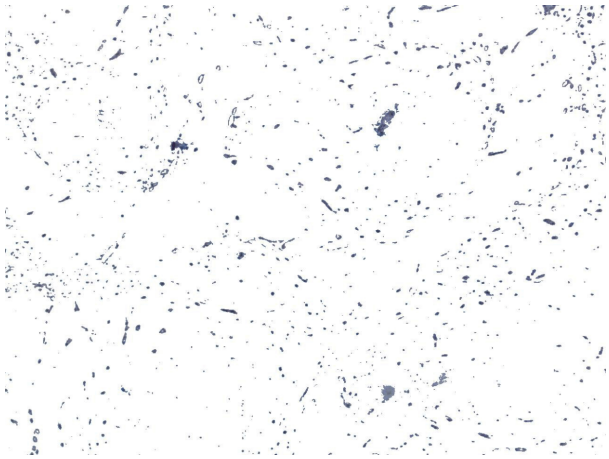

Figure 3 – ***The previous field (Figure 2), after colorimetric segmentation, based on the CD34+ areas, this image is now ready for fully automated analysis.***

Using immunohistochemical staining greatly enhances the potentials of colorimetric segmentation, since the DAB stain most of the time stands out clearly from the rest of the tissue. Performing this step has to be done manually, since there are notable differences between staining intensity, mostly due to fixation related issues or section thickness. The *second phase* is the measurement of the CD34+ areas. This has to be proceeded by the conversion of the image from 24-bit RGB format to 8-bit B&W format (Figure 4).

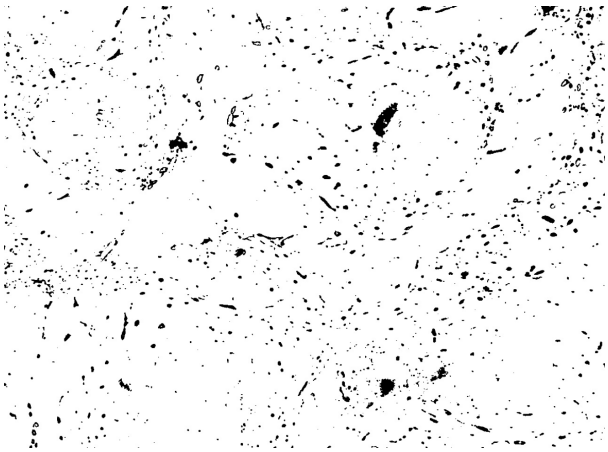

Figure 4 – ***8-Bit B&W image as the final step of the automated analysis.***

This latter format is ready for analysis, done with ImageJ's "Particle analysis" function with the proper settings. This phase can be fully automated so that the software automatically records the CD34+ areas in a spreadsheet next to the file name. In this way, the 200 images can be analyzed in less than 5 minutes and the results are readily introduced in a table.

As for statistical calculus, we used the Kolmogorov–Smirnov normality test and the Student's *t*-test. For both parameters, the average of the gathered data was used.

Results

A number of 10 digital pictures were taken from each case, represented by a digital slide, after immunohistochemical staining was performed for CD34. This resulted a total of 200 digital images, including the normal lymph node (19+1 cases).

The immunohistochemical reactions showed variable intensity of staining among the studied cases. However, the contrast of the staining of the vascular elements was strong enough to perform color-based segmentation and then digital morphometry. We performed HSB-based color segmentation. After this, we acquired two data sets for each type of lymphoma (DLBCL and FL) for each studied parameter. We performed the statistical analyses on these data. Taken separately the descriptive statistics of the two data sets are presented in Table 1 and the box plot graphics of the data sets are presented in Figures 5 and 6. On these images, can be noted that the FL group shows a much higher variability in terms of distribution regarding both parameters.

Table 1 – ***Descriptive statistics of the studied parameters in the two disease groups***

	Average vascular diameter [pixels]		EA area [%]	
	DLBCL	***FL***	***DLBCL***	***FL***
No. of values	*12*	*7*	*12*	*7*
Minimum	31	39	1.8	1.1
25% Percentile	36	40	2.8	3
Median	38	45	3.4	6.5
75% Percentile	45	64	4.7	10
Maximum	50	74	9.8	11
Mean	40	50	4	6.4
Std. Deviation	5.9	14	2.2	3.8
Std. Error	1.7	5.1	0.64	1.4

DLBCL – diffuse large B-cell lymphoma; FL – follicular lymphoma; EA – endothelial area; 1 pixel = 0.338 μm.

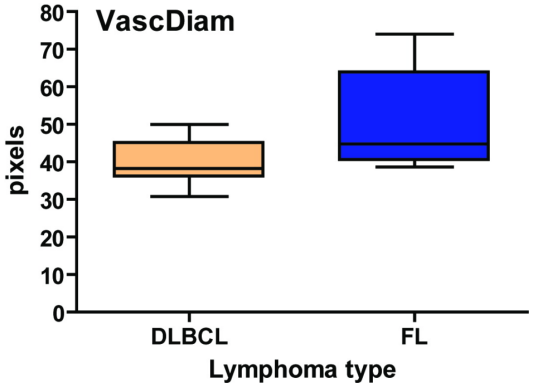

Figure 5 – ***Box plot diagram of the average vascular diameters (DLBCL – diffuse large B-cell lymphoma, FL – follicular lymphoma, 1 pixel = 0.338 μm).***

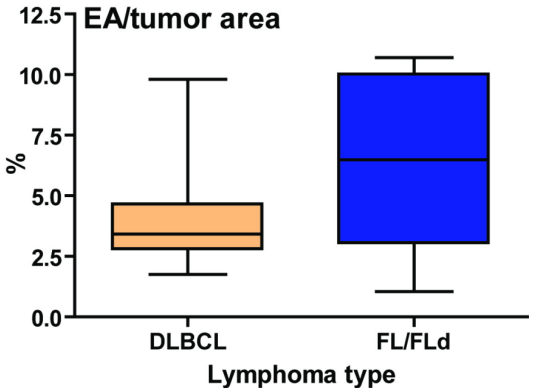

Figure 6 – ***Box plot diagram of the EA/tumor area ratio (DLBCL – diffuse large B-cell lymphoma, FL – follicular lymphoma).***

We compared the two data sets for the two disease types using unpaired Student's *t*-test, assuming Gaussian distribution. Interestingly, the mean average diameter of vascular structures showed significant differences between the two groups (p=0.0402); however, the EA/tumor area ratio did not show statistically significant difference (p=0.103). We speculated that this is because some of the cases from the FL group showed a partial diffuse histological aspect, cases nominated FLd, while others had nodular pattern (noted FLn). Upon dividing the FL group into the two distinct groups based on morphology alone (FLn and FLd) we found that there is statistically significant difference regarding the EA/tumor area ratio (p=0.02). According to large clinico-pathological studies FLd correlates with a more aggressive clinical course and has worse prognosis, therefore, it is of no surprise that angiogenesis in these types of FL differs significantly from those that have a clear-cut follicular pattern. In this regard those cases of follicular lymphoma that show a diffuse transformation might share angiogenetic characteristics with diffuse large B-cell lymphomas.

Furthermore, we noted that there are differences in vasculature patterns, without noticing significant bias towards any of the lymphoma types or subgroups. Since our morphometry method does not take into account the architecture of the microvasculature, only the areas that stain CD34 positive and the diameters of vessels, we consider this as yet an open question.

Discussion

Over the last few years a plethora of methods were developed and described to quantitatively assess microvascular density. These included the planimetric point-count method [9] modified to restrict counting to transversally cut microvessels occupying the reticulum intersection points [10] and the Chalkley method. Briefly, there are two major parameters that can be assessed to describe the vascularity of a given tumor: intratumoral microvascular density (MVD) and total microvascular density (TVA). They have slightly different techniques and both have been proven to have prognostic significance in various tumor types. Besides these parameters, there are other commonly used size- and shape-related parameters that include major axis length, minor axis length, perimeter, compactness, shape factor and Feret diameter. We used a previously successfully applied method [11, 12].

Although previous papers suggest taking images from the so-called „hot spots" from tumors [13, 15–17], we considered that taking more pictures from different areas of the tumor best describes the tumor's average microvascular density. In this case taking only from areas with high vascularity would give us an erroneous end-result of the microvascular density. In this regard, our method resembles to the TVA method, the only difference being that we took the images from the total area of the tumor. The tissue area marked by the CD34 corresponds to the endothelial area (EA).

A study shows that increased microvascular density in the bone marrow plays a significant role in the pathogenesis of hematological malignancies [19]. Microvascular density proved to have prognostic importance since it may alter the outcome of patients with lymphoma, in some studies higher microvascular density correlates with shorter survival [20, 21]. Farinha P *et al.* demonstrated that increased microvascular density in FL patients means high risk of transformation into DLBCL [22]. Angiogenesis is promoted by chemical mediators, such as angiogenic mediators and cytokines, both acting in paracrine or autocrine manner [23–25]. Understanding angiogenic mechanisms is important in the development of therapeutic targets in various types of non-Hodgkin lymphomas [26–28].

Conclusions

When performing the digital morphometry, we observed that taking more pictures from different areas of the tumor best describes the tumor's average microvascular density. This is particularly valid for lymphomas since we took pictures only from within the neoplastic areas. Therefore, we conclude that care must be taken in choosing the optimal areas one chooses to analyze.

Our study showed that there is no difference between DLBCL and FL (including all grades) regarding vascular density; however, vascular diameters are significantly different, being higher in FLs. This observation raises the need to further stratify these lymphomas and compare the different subgroups vascular parameters.

Nevertheless, increased microvascular density might play a significant role in the pathogenesis of hematological malignancies. Moreover, digital morphometry is an adequate and objective method in performing such studies.

Acknowledgments

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