Morphometric Angiogenesis Parameters for Indolent and Aggressive Non-Hodgkin’s Lymphoma

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There is much evidence about importance of angiogenesis in development and progression of solid tumors. The role of angiogenesis, as an indicator of higher malignant potential in non-Hodgkin’s lymphoma, is not clear at the moment. Morphometric characteristics of microvessels in lymph node sections, in previously untreated patients with small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) and diffuse large B-cell lymphoma (DLBCL), were studied and relationship between angiogenesis and histological malignancy grade of NHL was also evaluated. Lymph node biopsies samples of 30 newly diagnosed patients with SLL/CLL (n= 30) and DLBCL (n = 30) were studied. All samples were fixed in 10% buffered formalin solution and embedded in paraffin. Microvessels were visualized by immunohistochemical staining for anti F-8 antibody. In the area showing the most intense vascularization (i.e. the “hot spot”), microvessel density (MVD), total vascular area (TVA), as well as the size related parameters were estimated, by using image analysis program “analysSIS”. Number and size-related microvessels angiogenic morphometric parameters were statistically higher in group with DLBCL compared with SLL/CLL: MVD (p=0.002), TVA (p<0.0001), area (p<0.0001), perimeter (p<0.0001), minor axis length (p<0.0001) and major axis length (p<0.0001). It is to be noted that positive correlation existed between TVA and MVD in DLBCL and SLL/CLL. The present study supports the view that angiogenesis correlate with histological grade of NHL.

Key words: angiogenesis, NHL, morphometry, DLBCL, SLL/CLL.

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1. INTRODUCTION

In addition to its physiologic role in vascularization during ovulation, placentation, and embryogenesis, angiogenesis has been associated with the growth, dissemination, and metastasis of solid tumors (1, 2). Tumor angiogenesis pass through the same phase as physiological angiogenesis, but is uncontrolled, time unlimited and characterized by a 30-40 times greater proliferation of endothelial cells (3). When blood vessels grow uncontrolled, angiogenesis becomes pathologic and sustains the progression of many neoplastic and non-neoplastic diseases. It is essential in tumor progression, growth, invasiveness and metastasis, after the tumor switch from the avascular to vascular phase. Evidence supporting the importance of angiogenesis as an indicator of higher malignant potential of solid tumors are indisputable, but the clinical significance of this phenomenon in most hematological malignancies is still not clear (4). There is increasing evidence of the importance of angiogenesis in hematologic malignancies, indirectly implicating bone marrow angiogenesis in the pathophysiology and course of acute leukemia (5), chronic myeloid and lymphoblastic leukemia (6, 7, 8), myelodysplastic syndrome (4) and multiple myeloma (9, 10). Lymphoma growth and progression is potentiated by at least two distinct angiogenic mechanisms: autocrine stimulation of tumor cells via expression of vascular endothelial growth factor (VEGF) and VEGF receptors on lymphoma cells, as well as paracrine influences of proangiogenic tumors microenvironment on both local neovascular transformation and recruitment of circulating bone marrow-derived progenitors (11). Angiogenesis associated parameters are important prognosticators, and tumor blood vessels are an emerging target for therapy. In angiogenesis assays lymphoma cells show angiogenic properties. High levels of VEGF in blood and tissue are associated with adverse prognosis. VEGF and
VEGF receptors are also present in lymphoma cells. Therapy against endothelial vascular growth factors in animal models is effective and points to both tumor cells and the host endothelium as target (12). Neoangiogenesis process in cancer is influenced by the local microenvironment of the tumor itself (11).

MVD measures lymphoma neovascularization, which is generated in response to proangiogenic stromal cells, and infiltrating benign T/B lymphocytes within the tumor microenvironment. Therefore, data on MVD vary greatly among different studies due to the heterogeneity of lymphoma stroma, the range of cell surface markers used for staining and differences in scoring methodology (11).

2. MATERIAL AND METHODS

We assessed morphometric parameters of tumor angiogenesis in pretreatment lymph node biopsies of previously untreated randomly selected adult patients with SLL/CLL (n=30) and DLBCL (n=30), diagnosed and treated at the Department of Haematology University Clinical Centre Tuzla between 2001 and 2009. All tissue samples were pretreatment lymph node biopsies stored in the Department of Pathology at University Clinical Center Tuzla. All cases were reviewed by a pathologist with profound experience in the field of lymphoma, and classified according to the WHO classification. All specimens were previously fixed in formalin solution (10%) and embedded in paraffin. In histological sections of tissue sample 4 mm thick, an immunohistochemical analysis of a three-level immunoperoxidase with streptavidin was conducted. Histological sections were placed on organosilane pretreated glass slides and incubated overnight at 37 °C. Preparations were then deparaffinized and then incubated for 30 minutes in 1.5% hydrogen peroxideactivity, and then pretreated in citrate buffer (10 mM, pH 6.0) at 100°C for 15 minutes. Glass slides with histological specimens were appropriately stored at Shandon Sequenza Immunostaining Center where they carried out all degrees of incubation. Preincubation level, which lasted 15 minutes, with 10%-term normal bovine serum, was followed by the three-level immunoperoxidase procedure.

As a first, preparations were incubated with rabbit polyclonal antibody against Von Willebrand factor (F-8) at a dilution of 1:1200 (DAKO, Glostrup, Denmark). In the second instance, the incubation was performed for 30 minutes with biotin labeled by goat anti-rabbit antibody for polyclonal primary antibody. In the third instance, the incubation was performed by streptavidin-coated peroxidase, for 30 minutes. Rinses between incubation were carried out by phosphate buffered saline. Peroxidase activity was developed by 3,3-diaminobenzidin tetrahydrochloride and H2O2 as supstratum. Similar staining was performed with hematoxylin, and after dehydration, samples were mounted by Canadian balsam. For each tested sample and within the same procedure, histologic sections of tonsillar tissue were treated, as a positive control for the primary anti-F-8 antibody. A clear brown staining of cell membranes and cytoplasm endothelial cells was considered reliable positive reaction, along with the absence of nonspecific background staining. Immunocytochemical anti F-8 stained preparations were analyzed using Olympus BX51 microscope, using image analysis program (Analysis). In order to identify areas that showed the most intensive vascularization (so called hot spots), the entire clip of the lymph node was analyzed field by field, with an increase of 100 times. Selection of hot spots was based on a selection field that gives the impression the majority of anti F-8 stained microvessels.

Further analysis was performed under 200 times magnification. Hot spot was photographed and save in TIFF file. Any clearly stained endothelial cell or cluster was considered as a single countable microvessel, irrespective of the presence of a lumen. The outline of each microvessel was traced and the following morphometric parameters relating to microvessel number and caliber were estimated: the total count of microvessels in the hot spot (MVD), total vascular area (TVA), perimeter, major axis length and minor axis length. The variables entered into the statistical analysis were the total count of microvessels in the hot spot (MVD), the mean values of the above morphometric indices of the microvessels in the hot spot and total vascular area (TVA) (13).

3. STATISTICAL ANALYSIS

Statistical analysis was performed by using biomedical application software “© StatsDirect statistical software version 2.7.2”. Comparisons were made between the two groups, groups with SLL/CLL (n = 30) and DLBCL (n = 30). Numerical data were presented by the measures of central tendency and dispersion of appropriate measures. To test the hypothesis between two in-

![Figure 1. Immunohistochemical image of microvessels in SLL/CLL (IHH, F-8, 200X)](image)

![Figure 2. Immunohistochemical image of microvessels in DLBCL (IHH, F-8, 200X)](image)
dependent groups was used T-test or Mann-Whitney test if the observed discrepancy in the distribution of which is checked Kolmogorov-Smirnov test. For statistical significance values, p was chosen as the usual level of significance p < 0.05. Normality of distribution was tested by Kolmogorov-Smirnov test.

### 4. RESULTS

Analyzed samples were biopsied lymph nodes of patients with de novo SLL/CLL (n=30) and DLBCL (n=30). Microvessels were visualized by immunohistochemical staining for anti-F-8 antibody (Figure 1 and 2).

There was a significant difference in central tendency for the mean value of MVD (p <0.002) and TVA (p <0.0001). The mean value of MVD in the group with DLBCL was significantly higher than in the group with SLL/CLL (Table 1).

The mean value of the major and minor axis length, area and perimeter of microvessels were significantly higher in patients with DLBCL compared with a group of patients with SLL/CLL (p<0.001), (Table 2 and 3).

Positive correlations exist among TVA and MVD in patients with DLBCL (r=0.59, r²=0.34) (Figure 3), as well as, in patients with SLL/CLL (r=0.60, r² = 0.36) (Figure 4).

### 5. DISCUSSION

The evidences that confirms the importance of angiogenesis in hematologic malignancies are increasing. Angiogenesis is the subject of intense research in terms of basic processes during oncogenesis. The potential importance of angiogenesis in lymphomas in humans is based on the correlation of disease progression and increased angiogenic activity. The latest evidence, analyzing the angiogenic properties of tumor cells and vascular microenvironment, suggests that angiogenesis is highly relevant in many types of lymphoma (11). Previous findings suggest that B-NHL of intermediate and high grade malignancy match vascular phase, while tumors of low grade match avascular phase. According to research by Vacca et al. (14), angiogenesis is present in the lymph nodes in patients with intermediary (diffuse type) and highly malignant NHL (classified according to the classification of the Working Group), but not in NHL of low or intermediate degree of malignancy (follicular subtype). This correlates with the proliferation activity of B-NHL cells, due to previously proven increase of activity in tumors with high and intermediate level of malignancy.

Following numerous studies which showed that neovascularization is implicated in the pathogenesis of many hematologic malignancies, in this study we evaluated multiple morphometric parameters of neoangiogenesis (MVD, TVA and size related microvessels parameters) in two individual types of indolent and aggressive lymphomas: DLBCL and SLL/CLL. DLBCL is the most common type of aggressive lymphoma and often has an aggressive clinical course with high mortality rate in the first year after diagnosis with overall survival less than 50% (15).

We also focused on examining the differences in the pathological vascularization of lymph node in patients with DLBCL (representing aggressive lymphomas) and SLL/CLL (representing indolent lymphoma). Numerous studies have shown a positive correlation between tumor vascularization and the degree of malignancy, as well as the greater number of blood vessels in the lymph nodes of malignant lymphoma compare to reactive lymph node (16, 17, 18, 19).

However, other studies deny the association between MVD and NHL histological malignancy (20, 21). In addition, Mazur et al. (22) did not find a correlation between tumor MVD measured by expression of CD34 and NHL histological malignancy in lymph node samples from 40 patients with NHL classified according to REAL classification. Passalidou et al. in their study have also demonstrated increased vascularity in reactive lymph nodes compare to follicular lymphoma (23).

### Table 1. Microvessel count and total vascular area of microvessels in a hot spot of the lymph nodes in newly diagnosed patients SLL/CLL and DLBCL

<table>
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<tr>
<th>Parameters:</th>
<th>Groups tested</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>SLL/CLL (n=30)</td>
<td>DLBCL (n=30)</td>
</tr>
<tr>
<td>MVD</td>
<td>29.56 ± 10.02</td>
<td>40.86 ± 16.77</td>
</tr>
<tr>
<td>TVA (µ²)</td>
<td>6978.99 ± 2174.95</td>
<td>16104.10 ± 5595.78</td>
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**TABLE 1.** Microvessel count and total vascular area of microvessels in a hot spot of the lymph nodes in newly diagnosed patients SLL/CLL and DLBCL

### Table 2. The Major and minor axis length of microvessels in patients with newly diagnosed SLL/CLL and DLBCL

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<tr>
<td></td>
<td>SLL/CLL (n=30)</td>
<td>DLBCL (n=30)</td>
</tr>
<tr>
<td>Major axis length (µ)</td>
<td>23.63 ± 3.14</td>
<td>30.65 ± 6.52</td>
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<tr>
<td>Minor axis length (µ)</td>
<td>15.59 (14.00-16.99)</td>
<td>18.97 (1710-19.85)</td>
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**TABLE 2.** The Major and minor axis length of microvessels in patients with newly diagnosed SLL/CLL and DLBCL

### Table 3. Area and perimeter of microvessels in a lymph nodes of patients with newly diagnosed SLL/CLL and DLBCL

<table>
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<th>Groups tested</th>
<th>p-value</th>
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<tr>
<td></td>
<td>SLL/CLL (n=30)</td>
<td>DLBCL (n=30)</td>
</tr>
<tr>
<td>Area(µ²)</td>
<td>242.93 (185.95-301.62)</td>
<td>399.35 (313.48-435.98)</td>
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<tr>
<td>Perimeter (µ)</td>
<td>70.82 ± 9.22</td>
<td>92.18 ± 18.94</td>
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**TABLE 3.** Area and perimeter of microvessels in a lymph nodes of patients with newly diagnosed SLL/CLL and DLBCL

![FIGURE 3. TVA and MVD correlation in DLBCL](image)
Such findings questions the clinical relevance of the MVD (24), but also increase the necessity to explore correlation between MVD and NHL histological malignancy, in particular types of lymphoma.

In determining the significance of increased MVD, most studies describe the heterogeneous population, including a wide selection of NHL histological subtypes, and different therapeutic regimens. Phenotypic differences between vascular reactive lymph nodes, follicular lymphoma and DLBCL, indicate that the clinical significance of lymph node vascularity may be different in different histological entities.

According to some studies, the number of microvessels in NHL and CLL correlate with histological and clinical stage of disease (14, 17, 19, 25). These studies are in accordance to the above studies and represent a contribution to the research on the relationship of tumor vascularization and its degree of malignancy. It is proven that the number- and size-related microvessels angiogenic morphometric parameters were statistically higher in group with DLBCL compared to SLL/CLL: MVD (p=0.002), TSA (p=0.0001), area (p=0.0001), perimeter (p=0.0001), minor axis length (p=0.0001) and major axis length (p=0.0001).

According to our dates, a significantly higher number of microvessels, as well as other size related morphometric parameters of angiogenesis in the lymph nodes of patients with DLBCL, reflects an increased angiogenic potential in patients with DLBCL. The results of this study contribute to the view that antiangiogenic drugs have a place as adjuvant therapy in treatment of this form of NHL.

REFERENCES


FIGURE 4. TVA and MVD correlation in SLL/CLL