

## Density of intranodal lymphatics and VEGF-C expression in B-cell lymphoma and reactive lymph nodes

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**Abstract:** Lymphatic vasculature in solid tumors may serve as the pathway for metastatic spread of the cancer to the regional lymph nodes and to distant organs. Controversy still exists whether tumors metastasize through existing lymphatics or through newly formed vessels (lymphangiogenesis). The role of lymphangiogenesis in lymphoma spread and proliferation is not clearly established. VEGF-C is the most potent inducer of lymphangiogenesis. LYVE-1 was shown to be a specific marker for lymphatic vessels in normal and tumor tissue. The aim of the present study was the evaluation of lymph node LYVE-1-positive lymphatic sinus density (LSD) and VEGF-C expression in patients with non-Hodgkin's lymphoma (nHL) and in reactive lymph nodes. Sixty paraffin-embedded lymph nodes from newly diagnosed patients with B-cell nHL were evaluated. Twelve lymph node biopsy specimens from adult patients with reactive lymphonodulitis were used as controls. Sections of lymph nodes were stained immunohistochemically for LYVE-1 and VEGF-C. VEGF-C expression in lymph nodes of nHL patients was low and not significantly different from that in the control ( $p=0.6$ ). Moreover, VEGF-C expression did not differ significantly between aggressive and indolent lymphomas ( $p=0.53$ ). Similarly we did not find differences in LSD in aggressive nHL and in indolent nHL ( $p=0.49$ ). The mean LSD in reactive lymph nodes was higher than in nHL ( $p=0.03$ ). Only in 2 out of 12 reactive lymph nodes LYVE-1-positive vessels were absent. In all groups we demonstrated a strong positive correlation between VEGF-C and LYVE-1 expression ( $p=0.0001$ ). Higher LSD in reactive lymph nodes as compared to those of nHL patients suggests that lymphoma proliferation leads to the destruction of the existing lymphatics rather than to lymphangiogenesis within lymph nodes. NHL are not associated with increased expression of VEGF-C nor increased LYVE-1-positive lymphatic sinuses density within lymph nodes. ([www.cm-uj.krakow.pl/FHC](http://www.cm-uj.krakow.pl/FHC))

**Key words:** Non-Hodgkin's lymphoma - LYVE-1 - VEGF-C - Lymphangiogenesis

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### Introduction

Lymphangiogenesis is a formation of new lymphatic vessels from preexisting capillaries. The development of lymphatic vessels depends upon interactions between lymphatic endothelium and signaling molecules derived from serum and extracellular matrix. Vascular endothelial growth factors (VEGFs) family of glycoproteins is essential for angiogenesis and lymphangiogenesis. Among these proteins, VEGF-C and VEGF-D are the most potent inducers of lymphangiogenesis. VEGF-C stimulates lymphangiogenesis by binding to VEGFR-2 and VEGFR-3 receptors on lymphatic endothelial cells

[13, 29, 30]. Increased VEGF-C expression correlates with increased risk of metastasis in regional lymph nodes in many solid tumors.

The role of lymphangiogenesis in the development of cancer and tumor metastasis is, however, still controversial. It has been proposed that tumors may stimulate lymphatic endothelium proliferation in analogous way as in tumor angiogenesis. Animal model studies have confirmed that VEGF-C produced by tumor cells promotes lymphangiogenesis and lymph nodes metastasis [15, 29]. Non-Hodgkin's lymphomas (nHL) can spread via blood vasculature or lymphatics. Lymphoma cells may infiltrate the lymph nodes, surrounding tissue or migrate to other organs. Angiogenesis in haematological malignancies was a subject of intensive research in the last years. Increased microvessel density (MVD) was demonstrated in nHL [17, 20]. However the relationships between MVD and histologic subtypes of lym-

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phoma are not clear. Elevated serum levels of proangiogenic cytokines (*i.e.* VEGF) are the independent prognostic factor in nHL [24]. Data concerning the role of lymphangiogenesis in nHL are very limited. Salven *et al.* showed low VEGF-C mRNA in lymphoma cells [23]. Studies of tumor lymphangiogenesis have been hampered by the lack of specific and reliable markers for lymphatic endothelium. LYVE-1, a CD44 homologue, and hyaluronan (HA) receptor predominantly expressed on lymphatic endothelium was shown to be a specific marker for lymphatic vessel in normal and tumor tissues. It is also abundant in lymph nodes, especially in high endothelial venules [1, 34]. LYVE-1 plays a crucial role not only in HA binding and metabolism but also in leukocyte trafficking within lymphatic vessels and lymph nodes. LYVE-1 interaction with HA may facilitate tumor cells attachment and enhance lymphoma growth and dissemination.

The aim of the present study was the evaluation of LYVE-1-positive lymphatic sinus density (LSD) and VEGF-C expression in lymph nodes of patients with nHL and in control reactive lymph nodes.

## Materials and methods

Sixty paraffin-embedded lymph nodes from newly diagnosed patients with B-cell nHL were evaluated (22 aggressive and 38 indolent nHL). In addition, 12 lymph node biopsy specimens from adult patients with reactive lymphonodulitis were used as controls. The lymphoma group consisted of 24 females and 36 males, median age 62 years (range 27-70 years). Diagnosis of lymphoma was made according REAL/WHO classification [9]. Aggressive nHL group consisted of 11 cases of diffuse large B-cell lymphoma, 4 cases of follicular lymphoma G3, 3 cases of mantle cell lymphoma, 2 cases of Burkitt lymphoma and 2 cases of lymphoblastic lymphoma. Indolent nHL group consisted of 20 cases of follicular lymphoma G1/G2, 12 cases of small lymphocytic lymphoma, 4 cases of nodal marginal zone lymphoma and 2 cases of lymphoplasmacytoid lymphoma.

Lymph node sections were stained immunohistochemically for LYVE-1 and VEGF-C. The studied samples were fixed in 10% buffered formalin and subsequently embedded in paraffin. The sections were stained with hematoxylin and eosin and evaluated histopathologically. Deparaffinised sections were pre-treated with Target Retrieval Solution (DakoCytomation, Denmark) at 95°C for 20 min. Following a wash in Tris-buffered saline (TBS), they were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min, washed in distilled H<sub>2</sub>O (10 min) and PBS (5 min), and incubated with mouse monoclonal antibodies against LYVE-1 (RELIATech GmbH, Germany) (diluted 1:200) or against VEGF-C goat polyclonal antibody (C-20, catalog no. sc-1881; Santa Cruz Biotechnology, USA) for 60 min at room temperature. For all slides, a wash in TBS was followed by treatment with peroxidase-labeled polymer conjugated to goat anti-rabbit, anti-mouse or anti-goat immunoglobulins (Universal LSAB<sup>TM</sup>+ Kit; DakoCytomation, Denmark) for 30 min at room temperature. Immunostaining was visualized with diaminobenzidine tetrahydrochloride (DAB; DakoCytomation, Denmark) and then the sections were counterstained with hematoxylin. In each case the negative control was included using Primary Negative Control (DakoCytomation, Denmark) [11, 26].

For the evaluation of lymphatic sinus density (LSD) within lymph nodes, slides were scanned in the light microscope (Olympus BX-41) at × 40 magnification and three areas of maximal LVD,

**Table 1.** Evaluation of the reaction results using the ImmunoReactive Score (IRS) scale

	% positive cells		Intensity of the reaction
0	no positive cells	0	no positive reaction
1	<10% positive cells	1	faint colour reaction
2	10-50% positive cells	2	moderate colour reaction
3	51-80% positive cells	3	intense colour reaction
4	>80% positive cells		

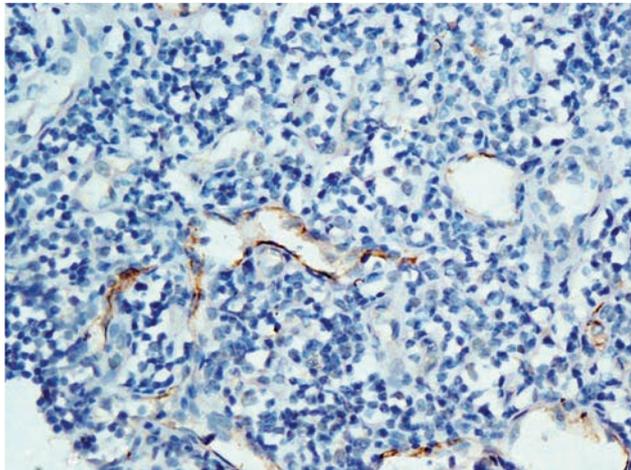
so-called "hot spots" were identified. In each hot spot (each field representing an area of 0.375 mm<sup>2</sup>), lymphatic sinuses were counted at × 400 magnification by two independent investigators.

Intensity of immunocytochemical expression of VEGF-C was evaluated using the ImmunoReactive Score (IRS) according to Remmele and Stenger [19]. The applied scale took into account both intensity of the colour reaction and percentage of cells, which exhibited the positive reaction (Table 1). The final result represented the product of the two parameters and its value ranged from 0 to 12. Statistical analysis was carried out using the Mann-Whitney U-test, Anova Kruskal Wallis test and Spearman test. Differences were considered statistically significant at p<0.05.

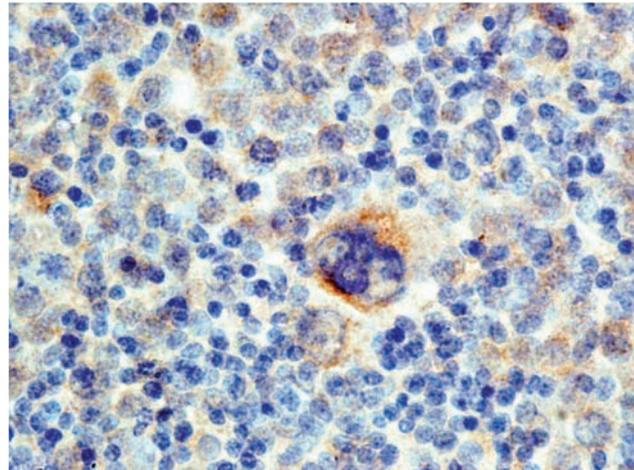
## Results

VEGF-C was expressed in the cytoplasm of lymphoma cells infiltrating lymph nodes and in lymphocytes in reactive lymphonodulitis. We demonstrated LYVE-1-positive reaction on endothelium of lymphatic sinuses and some high endothelial venules in lymph nodes irrespectively of initial diagnosis (lymphoma or reactive lymph nodes) (Figs. 1, 2). Statistical analysis showed that VEGF-C expression in lymph nodes of nHL patients was low and not significantly different from that in the control: the mean IRS 2 (range 0-6) and 2 (range 0-6) respectively (p=0.6). Moreover, VEGF-C expression did not differ significantly between aggressive and indolent lymphomas: mean IRS 2 (0-6) and 1, respectively (0-6) (p=0.53). Similarly, we did not find differences in LSD in aggressive nHL - 3 (0-27) and in indolent nHL - 2.6 (0-11) (p=0.49). The mean LSD in reactive lymph nodes was higher than in nodes of nHL patients: 8.4 (range 0 - 21) and 2.8 (range 0-27), respectively (p=0.03). Only in 2 out of 12 reactive lymph node specimens LYVE-1-positive vessels were absent. In all groups we demonstrated strong positive correlation between VEGF-C and LYVE-1 expression (p= 0.0001).

We correlated VEGF-C and LSD visualized by LYVE-1 expression with some well known parameters of prognosis in nHL such as histological subtypes (aggressive versus indolent), clinical stage of disease and dehydrogenase lactate (LDH) activity. We found no statistically significant correlations for these parameters. The association between LSD, VEGF-C and clinicopathological data are shown in Table 2.



**Fig. 1.** LYVE-1-positive lymphatic sinuses in reactive lymph node.  $\times 200$ .



**Fig. 2.** VEGF-C expression in lymphoma lymph node (Large B-cell lymphoma).  $\times 400$ .

## Discussion

The lymphatic vasculature may serve as a pathway for metastatic spread of cancer to the regional lymph nodes and to distant organs. Controversy still exists whether tumors metastasize through the existing lymphatics or through newly formed vessels. Recently, LYVE-1 (lymphatic endothelium hyaluronan receptor) has been identified as a powerful marker for lymphatic endothelium. LYVE-1 is also expressed by a subset of infiltrating macrophages found in tumors, liver blood sinusoids, and high endothelial venules in lymph nodes and spleen [4, 12, 34]. Little is known about the biology of the lymphatics in tumors and the regulation of tumor induced lymphangiogenesis. Several studies were focused on assessment of lymphatic vessel density in and around tumors. The data concerning the prognostic significance of intra- and peritumoral lymphangiogenesis are confusing. There is increasing evidence based on animal model that metastatic spread occurs via peritumoral rather than intratumoral lymphatics [18]. Moreover, intratumoral lymphatics are not features of all tumor types. Williams *et al.* [33] reported the absence of lymphatic vessels within both tumor and surrounding areas. In solid tumors, immunohistochemical studies with anti-LYVE-1 antibodies resulted in different findings in various tumor types. Trojan *et al.* [31] reported lack of lymphangiogenesis in prostate cancer. LYVE-1-positive lymphatic vessels were detected at the tumor periphery of endometrial and lung cancers but not within the main tumor mass [14]. Peritumoral but not intratumoral LYVE-1-positive vessels were identified in the majority of breast carcinoma cases [3, 32]. In head and neck squamous cell carcinoma, increased number of intratumoral lymphatics was associated with the status of regional lymph node metastasis. The presence of

intratumoral lymphatic vessels was also shown in melanoma [6, 16, 27].

VEGF-C enhances lymphangiogenesis and promotes metastasis via lymphatic vessels. Several studies have shown a correlation between VEGF-C expression and lymph node metastases. There is convincing evidence that VEGF-C expression is upregulated in many solid tumors [10, 28]. VEGF-C is expressed not only on tumor cells but also on stromal cells including macrophages. Inflammation or cancer induce recruitment of macrophages and overproduction of VEGF-C [25]. Furthermore, VEGF-C itself may induce macrophage chemotaxis [27, 28]

Non-Hodgkin's lymphomas are heterogeneous group of lymphoid malignancies with different clinical course. NHLs represent the malignant counterparts of normal lymphocytes, arrested at specific stages of maturation. Lymphocytes enter lymphatic capillaries and migrate through the lymphatic vessels to the lymph nodes. Indolent lymphomas related to small resting lymphocytes usually are disseminated at presentation, whereas aggressive lymphomas related to activated lymphocytes are often initially localized [2]. The role of lymphangiogenesis in lymphoma dissemination remains to be determined. In murine model of Burkitt's lymphoma, overexpression of c-Myc in B lymphocytes resulted in increasing density of lymphatics (sinuses and vessels) within lymph nodes [21]. The importance of CD44 in the biology of lymphoma is well established. In diffuse large B cell lymphoma, increased expression of CD44 correlates with lymphoma dissemination [7, 8]. Structural and functional similarities between CD44 and LYVE-1 prompted us to investigate LYVE-1 expression within lymph nodes in lymphoma patients. Our study has been focused on the assessment of lymphatic sinus density in lymph nodes by LYVE-1 immunohistochem-

**Table 2.** Association between LVD, VEGF-C and clinicopathological data

	No. of patients total	No. of patients		p	No. of patients		p
		LSD=0	LSD > 0		VEGF-C IRS=0	VEGF-C IRS > 0	
Stage of lymphoma							
I/II	12	7	5	NS	6	6	NS
III/IV	48	27	21		27	21	
Histology of the lymph node							
Indolent nHL	38	2	18	] NS* ] <0.05**	20	18	NS
Aggressive nHL	22	14	8		13	9	
Reactive	12	2	10		4	8	
LDH							
Normal	24	11	13	NS	12	12	NS
Elevated	36	23	13		21	15	

LSD - lymphatic sinus density; LDH - lactate dehydrogenase; \*indolent versus aggressive nHL; \*\*all nHL versus reactive lymph nodes

istry and on VEGF-C expression in lymph nodes. We analyzed LSD and VEGF-C expression in a variety of histological subtypes and clinical aggressiveness of lymphoma. We demonstrated that expression of VEGF-C, the most potent cytokine stimulating lymphangiogenesis, was not increased in lymph nodes of lymphoma patients when compared to reactive lymph nodes. According to Salven *et al.* [23], low expression of VEGF-C in nHL may reflect the cell-specific pattern of expression of the VEGF-C gene in the corresponding normal cells. We found no correlation between VEGF-C expression and clinical stage of lymphoma or widely used prognostic factor such as LDH activity. On the other hand, in all groups we observed a strong positive correlation between VEGF-C and LSD. This observation confirms the role of VEGF-C as an inducer of the development of lymphatic vasculature. Higher LSD in reactive lymph nodes than in nHL lymph nodes suggests that lymphoma proliferation leads to destruction of the existing lymphatics rather than to lymphangiogenesis within lymph nodes. Moreover, increased LSD with relatively low VEGF-C expression in reactive lymph nodes indicates other mechanisms stimulating proliferation of lymphatic endothelium. Recently Cursiefen *et al.* [5] have demonstrated that inflammatory lymphangiogenesis may be indirectly stimulated by VEGF-A via macrophage recruitment. In conclusion, nHLs are not associated with increased expression of VEGF-C or increased LYVE-1-positive lymphatic sinus density within lymph nodes.

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