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CLINICAL SIGNIFICANCE OF VEGF-A AND MICROVESSEL DENSITY IN DIFFUSE LARGE B-CELL LYMPHOMA AND LOW-GRADE FOLLICULAR LYMPHOMA

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SUMMARY – Angiogenesis is essential for the development, growth and progression of tumors. Although vascular endothelial growth factor (VEGF) is a well-known proangiogenic factor, its impact on lymphoma has not yet been fully clarified. The aim of this study was to evaluate VEGF-A expression and microvessel density (MVD) in aggressive lymphoma such as diffuse large B-cell lymphoma (DLBCL), in indolent lymphomas such as low-grade follicular lymphoma (FL), and in lymph node reactive follicular hyperplasia (FH). In 80 prospective and retrospective cases (30 DLBCL, 30 FL and 20 FH), CD31 was analyzed by immunohistochemical staining assessing density of blood vessels, as well as the total number of CD31 positive endothelial cells. The results were compared with relevant clinical data. MVD was 85% in FH, followed by 60% in DLBCL and 43% in low-grade FL. VEGF-A was significantly higher in DLBCL than in low-grade FL and FH. A statistically significant association of MVD and VEGF-A with the International Prognostic Index (IPI) was found in DLBCL. High MVD and VEGF-A expression was observed in DLBCL patients with high IPI, while there was no statistically significant association between MVD and VEGF-A with the Follicular Lymphoma International Prognostic Index in low-grade FL. Our results suggested an important relationship between angiogenesis and high-grade lymphoma.

Key words: *Vascular endothelial growth factor A; Lymphoma, non-Hodgkin; Lymphoma, large B-cell, diffuse; Lymphoma, follicular; Lymph nodes; Hyperplasia*

Introduction

Diffuse large B-cell lymphoma not otherwise specified (DLBCL) is the most common type of lymphoid tumor worldwide[1,2], and displays striking heterogeneity at clinical, genetic, and molecular levels. DLBCL was included both in the Revised European American Lymphoma (REAL) and World Health Organization (WHO) classifications aiming to describe all malignant lymphomas characterized by the large size of neoplastic cells, B-cell derivation, aggressive clinical presentation, and need for highly effective chemotherapy regimens[3,4].

Follicular lymphoma (FL) is the second most common non-Hodgkin's lymphoma. This disease affects lymph nodes, and 50% of patients present with bone marrow infiltration. However, the mechanisms involved in dissemination of the disease are still unclear[5].

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FL is divided into three grades. Grades 1 and 2 are considered as lymphoma of low-grade malignancy[6]. Angiogenesis is the formation of new capillaries by outgrowth of endothelial cells from preexisting blood vessels[7].

The Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor (VEGF/VEGFR) pathway is a key mediator of angiogenesis and VEGF-A acts as a potent tumor angiogenic factor. VEGF-A stimulates the growth of new blood vessels that provide tumors with oxygen and nutrients, which in turn determine the proliferative activity of the tumor. Expression of VEGF-A has been shown to be regulated at the transcriptional and translational levels[8,9].

VEGF-A mediated angiogenesis has received considerable attention in the context of solid neoplasia, particularly with the clinical use of anti-VEGF-A antibodies and small molecule VEGFR inhibitors. More recently, the concept of tumor vascularity has been applied to hematolymphoid neoplasia, with studies quantifying microvessel density (MVD) in a variety of lymphomas[10,11].

VEGF-A-mediated signaling has at least two potential roles in DLBCL, i.e. potentiation of angiogenesis, and potentiation of lymphoma cell proliferation and/or survival induced by autocrine VEGFR-mediated signaling[10-12].

Given the rapidly increasing availability of a variety of pharmaceuticals aimed at the VEGF-A pathway, the role of angiogenesis and VEGF-A signaling in DLBCL may be of great interest[10].

In non-Hodgkin's lymphomas, expression of angiogenic factors in cell lines[14] and neoplastic tissue[15-17] has been demonstrated. Although these studies suggest a role for angiogenesis in lymphomas, several questions remain unanswered. In addition, determination of MVD as a measure of the degree of angiogenesis is also one of the most examined parameters for angiogenesis in cancers[13]. It is not clear whether high MVD is associated with more aggressive lymphomas.

The aim of this study was to evaluate VEGF-A expression and MVD through expression of CD31 in endothelial cells in aggressive lymphomas such as DLBCL, in indolent lymphomas such as FL, and in lymph node reactive follicular hyperplasia (FH).

Furthermore, we analyzed the correlation of VEGF-A expression and MVD with the International Prognostic Index (IPI) in patients with DLBCL and Follicular Lymphoma International Prognostic Index (FLIPI) in patients with low-grade FL.

Materials and Methods

In our study, we evaluated VEGF-A and CD31 expression in 30 DLBCL, 30 FL and 20 FH cases diagnosed at the Institutes of Pathology, Faculty of Medicine, University of Prishtina and Faculty of Medicine, St. Cyril and Methodius University in Skopje.

Immunohistochemistry

The slides were deparaffinized and rehydrated using standard procedures. Microwave heating was used for antigen retrieval and slides were then IHC stained using VEGF-A (A-20, 1:150 dilution; Santa Cruz Biotechnology Inc., USA, and CD31 (Clone JC70A, RTU; DAKO, Denmark) antibodies.

Protein expression of VEGF-A observed mainly in the cytoplasm of tumor cells was scored by combining the quantity score (percentage of positive stained cells) with the staining intensity score. All slides were analyzed independently by three pathologists. The quantity score ranged from 0 to 4: 0 = no immunoreactivity; 1 = <25% cells stained; 2 = 26%-50% cells stained; 3 = 51%-75% cells stained; and 4 = >76% cells stained. Then, scores 1-4 were clustered into two groups: group I (scores 1 and 2) and group II (scores 3 and 4). The staining intensity was scored as: 0 (negative), 1 (weak positivity, seen at X400 magnification), 2 (moderate, seen at X100 magnification), and 3 (strong, seen at X40 magnification). The final score was obtained by multiplying the groups of quantity score and intensity, as follows: 0 = no immunoreactivity; 1 = low positivity (1%-50% of cells stained and seen at X400); 2 = moderate positivity (1%-50% cells stained and seen at X40x or >50% of cells stained and seen at X400); and 3 = high positivity (51%-100% cells stained and seen at X40).

Microvessel counting was used for angiogenesis assessment. CD31 immunostained tumor sections were scanned at low power magnification (X100) to identify the areas with highest vascular density, so called 'hotspots'; then MVD was calculated as the mean value of CD31-positive cells and cell clusters in five hot spots *per* section evaluated at X200 magnification. Subse-

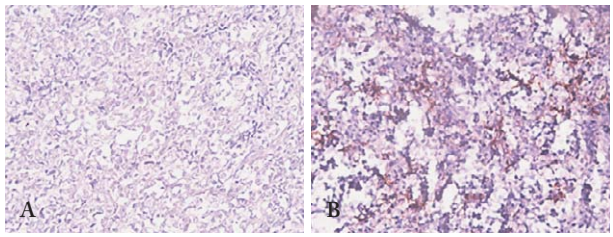

Fig. 1. VEGF-A expression: (A) no immunoreactivity; and (B) high immunoreactivity at X40 magnification.

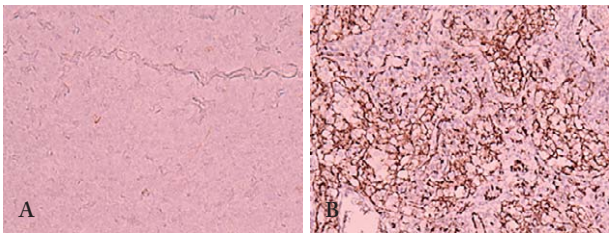

Fig. 2. CD31 staining: (A) low microvessel density; and (B) high microvessel density at X40 magnification.

Table 1. Number of cases per group and expression of vascular endothelial growth factor-A

VEGF-A	Score ranges			
	0	1	2	3
DLBCL	1	5	17	7
Low-grade FL	5	4	19	2
FH	5	5	10	0

VEGF-A = vascular endothelial growth factor-A; DLBCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; FH = follicular hyperplasia

Table 2. Number of cases per group and expression of microvessel density

	MVD	
	Low	High
FL	17	13
DLBCL	12	18
FH	4	16

χ^2=6.688, DF=2, p-value 0.035
MVD = microvessel density; DLBCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; FH = follicular hyperplasia

quently, the MVD median value was used to classify each group of tumors in 'high' and 'low' MVD, by three independent observers to obtain consensus of categorization. The cut-off to categorize 'low' and 'high' MVD was a mean value of 10 blood vessels.

Prognostic index

The scoring systems, known as the IPI for DLBCL and FLIPI for FL, were used to compare results with clinical data. Three risk groups were categorized using the IPI and FLIPI scoring system: low risk, intermediate risk, and high risk[18,19].

Statistical analysis

Univariate analysis was performed to assess the percentage distribution and other main characteristics of single variables used in testing the hypotheses. The χ^2-test was used to test differences and bivariate analysis to test correlation between categorical variables. All hypotheses were tested at the 0.05 level of significance.

Results

Expression of VEGF-A in DLBCL was scored as follows: no immunoreactivity, 1 (3%) case; low positivity, 5 (17%) cases; moderate positivity 17 (57%) cases; and high positivity, 7 (23%) cases (Fig. 1). However, in FL, VEGF-A expression was scored as follows: no immunoreactivity, 5 (17%) cases; low positivity, 4 (13%) cases; moderate positivity, 19 (63%) cases; and high positivity, 2 cases (7%). Lastly, in FH, 5 (25%) cases showed low positivity and 15 (50%) cases showed moderate positivity, while no cases showed high positivity (Table 1).

Positive staining for CD31 was detectable in the membrane of endothelial cells in the stroma. Of 30 DLBCL cases, 18 (60%) showed high MVD and 12 (40%) showed low MVD, whereas in FL 13 (43%) cases showed high MVD and 17 (57%) cases showed low MVD. Control group (FH) was found to have a very high expression of MVD, as 16 (80%) cases showed high MVD and only 4 (20%) cases showed low MVD (Fig. 2). The p-values obtained by the χ^2-test yielded statistically significant differences between the two groups of MVD (χ^2=6.688, df=2, p=0.035) (Table 2).

Expression of VEGF-A was compared for the relationship with clinical data, i.e. prognostic index (IPI for DLBCL and FLIPI for FL). One DLBCL case without staining also had low IPI, 5 of 7 cases with high VEGF-A immunoreactivity had high IPI, an-

Table 3. Correlation between International Prognostic Index in DLBCL and expression of CD31

Variable	IPI		
CD31	Low	Intermediate	High
Low	8	3	1
High	0	13	5

χ^2=16.372, DF=2, p-value 0.000, Spearman correlation=0.633
DLBCL = diffuse large B-cell lymphoma; IPI = International Prognostic Index

Table 4. Correlation between International Prognostic Index in DLBCL and VEGF-A

	IPI		
VEGF-A	Low	Intermediate	High
0	1	0	0
1	4	1	0
2	4	12	3
3	0	2	5

DLBCL = diffuse large B-cell lymphoma; IPI = International Prognostic Index; VEGF-A = vascular endothelial growth factor-A; study results showed highly statistically significant correlation between VEGF-A and IPI in DLBCL

other 2 cases had intermediate IPI, and no case with high immunoreactivity had high IPI (Table 3). However, 4 out of 5 FL cases with no VEGF-A immunoreactivity showed low FLIPI, and 1 case had intermediate FLIPI. Of 2 FL cases with high expression of VEGF-A, 1 case had high and intermedicate FLIPI each.

Expression of MVD was also compared for the relationship with clinical data, i.e. prognostic index (IPI for DLBCL and FLIPI for FL). Out of 17 cases of FL with low MVD, 8 showed low, 5 intermediate and 4 high FLIPI. Four of 13 cases with high MVD showed low FLIPI, 7 intermediate FLIPI, and only 2 showed high FLIPI. Based on the test performed, there were no statistically significant differences between MVD and FLIPI (χ^2=1.833, df=2, p=0.4).

In DLBCL, different results were noted. Eight of 12 cases with low MVD showed low IPI, 3 cases intermediate, and only 1 case high IPI. No case with high MVD was in the category of low IPI, however, 13 cases were in the intermediate and 5 in the high IPI category. Study results showed highly statistically significant correlation between MVD and IPI in DLBCL (χ^2=16.372, df=2, p-=0.000, Spearman correlation=0.633) (Table 4).

Discussion

Angiogenesis is a multistep process playing a crucial role in the progression and metastasis of various tumors, including those of visceral organs and hematolymphoid malignancies[20]. Tumor angiogenesis has been well documented both in experimental and clinical studies, and the degree of angiogenesis was closely associated with tumor progression and shorter patient survival in many types of cancer[21,22]. Non-Hodgkin's lymphomas are a diverse group of lymphoproliferative neoplasms with a variable clinical behavior and prognosis. Currently, lymphomas are classified on the basis of morphology, immunology, genetic, and clinical features. The clinical stage of the lymphoma is the most important prognostic factor, apart from the histologic grade (indolent *versus* aggressive)[20].

Microvessel density measures lymphoma neovascularity generated in response to tumor cells, proangiogenic stromal cells, and infiltrating benign T/B lymphocytes and myeloid cells within the tumor microenvironment. Therefore, MVDs 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. The clinical predictive value of MVD with respect to underlying lymphoma subtypes remains unclear[23].

In general, MVD scores trend highest in aggressive subtypes of lymphoma, compared with lower ones in indolent lymphoma. Gratzinger *et al*. report that average MVDs did correlate with the intensity of VEGF staining in a statistically significant manner[24]. In another study, increasing MVD was found to be a poor prognostic indicator for overall but not progression-free survival, and was independent of the IPI[25].

Koster *et al*.[11] found that in FL, high MVD as a variable of increased tumor vascularization was associated with a significantly more favorable outcome in terms of both progression-free and overall survival[26]. Positive association between MVD and patient outcome is in contrast with the notion that in hematologic malignancies, as well as in solid tumors the increase of angiogenesis-associated variables is related to adverse prognosis[15,22,28]. Aggarwal *et al*.[20] found higher MVD in aggressive lymphoma such as peripheral T-

cell lymphoma and DLBCL, and low MVD in low-grade lymphomas[2].

In our study, VEGF-A and CD31 staining were evaluated as markers of tumor vascularity in lymph nodes, showing significant difference in terms of MVD between high-grade lymphoma (DLBCL), which demonstrated high MVD, and low-grade FL, which showed low MVD.

Ganjo *et al.*[29,30] report that antiangiogenic drugs have modest clinical activity in lymphomas as a single agent. Our results may suggest that antiangiogenic therapy could be active only in high-grade lymphomas expressing stronger VEGF.

Conclusion

Neoangiogenesis has been increasingly recognized to play potentially important pathogenic roles in lymphomagenesis. We can conclude that our results support the hypothesis that there is important relationship between angiogenesis and high-grade lymphoma. Also, there is significant correlation with clinical data.

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Sažetak

KLINIČKA VAŽNOST VEGF-A I MIKROŽILNE GUSTOĆE U DIFUZNOM LIMFOMU VELIKIH B-STANICA I FOLIKULARNOM LIMFOMU NISKOG STUPNJA

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Angiogeneza je bitna za razvoj, rast i progresiju tumora. Iako je vaskularni endotelni faktor rasta (VEGF) dobro poznati proangiogeni čimbenik, njegov utjecaj na limform nije u potpunosti razjašnjen. Cilj ovoga istraživanja bio je procijeniti izraženost VEGF-A i mikrožilnu gustoću (MVD) kod agresivnog limfoma kao što je difuzni limfom velikih B-stanica (DLBCL), kod indolentnih limfoma kao što je folikularni limfom niskog stupnja (FL) i kod reaktivne folikularne hiperplazije limfnih čvorova (FH). Analiza CD31 provedena je u 80 prospektivnih i retrospektivnih slučajeva (30 DLBCL, 30 FL i 20 FH) imunohistokemijskim bojenjem za procjenu gustoće krvnih žila, kao i ukupnog broja endotelnih stanica pozitivnih na CD31. Rezultati su uspoređeni s relevantnim kliničkim podacima. MVD je bila 85% kod FH, 60% kod DLBCL i 43% kod FL niskog stupnja. VEGF-A je bio značajno viši u DLBCL u odnosu na FL niskog stupnja i FH. Statistički značajna udruženost MVD i VEGF-A s Internacionalnim prognostičkim indeksom (IPI) utvrđena je kod DLBCL. Visoka izraženost MVD i VEGF-A zabilježena je u bolesnika s DLBCL s visokim IPI, dok nije bilo statistički značajne udruženosti MVD i VEGF-A s Internacionalnim prognostičkim indeksom za folikularni limfom kod FL niskog stupnja. Rezultati ovog istraživanja ukazuju na važan odnos između angiogeneze i limfoma visokog stupnja.

Ključne riječi: *Vaskularni endotel, faktor rasta A; Limfom, ne-Hodgkin; Limfom velikih B-stanica, difuzni; Limfom, folikularni; Limfni* čvorovi*; Hiperplazija*