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Prognostic importance of thrombospondin-1, VEGF, PDGFR-β in diffuse large B-cell lymphoma

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ABSTRACT

Aim: In this study, we aimed to investigate the relationship between the staining rates of thrombospondin-1, VEGF, and PDGFR-in tissue preparations in patients diagnosed with DLBCL and their clinical features at the time of diagnosis, and response to treatment and prognosis.

Material and Method: A total of 44 patients with a diagnosis of DLBCL and 13 patients diagnosed with control reactive lymphadenopathy were included in this study. After immunohistochemical staining of the pathology preparations of the patient and control groups with VEGF, PDGFR-β and thrombospondin-1 stains, the clinical characteristics of the patients and the relationship between survival analysis and staining rates were statistically analyzed.

Results: When the patients were compared with the control group in terms of VEGF, PDGFR-β, and thrombospondin-1 staining rates, we found that staining with PDGFR-β was lower in patients (p=0.009). Although it was not statistically significant for PDGFR-β, it was observed that 5-year OS and PFS values were low in patients with high levels of expression, on the contrary, 5-year OS was low in patients with high thrombospondin staining rate. A negative correlation was observed between thrombospondin-1 and PDGFR-β (p=0.003, r=-0.440).

Conclusion: As a result, although no relationship was found between VEGF and survival in our study, it was observed that PDGFR-β and thrombospondin-1 were effective in prognosis. A negative correlation was observed between thrombospondin-1 and PDGFR-β.

Keywords: Diffuse large B cell lymphoma, thrombospondin-1, VEGF, PDGFR-β, prognosis

INTRODUCTION

Diffuse large B cell Lymphoma (DLBCL) is the most common subtype of Non-Hodgkin Lymphoma (NHL)(1). Angiogenesis is regulated by the balance between angiogenic and anti-angiogenic factors. Microvascular density and tumor angiogenesis were found to be poor prognostic factors in patients with DLBCL receiving anthracycline-based chemotherapy (2). VEGF (Vascular endothelial growth factor), and PDGF (Platelet derivated growth factor) are the leading angiogenic factors, and thrombospondin-1 is the leading antiangiogenic factor. In studies performed with serum VEGF levels of patients with a diagnosis of DLBCL, those with high serum VEGF levels were found to be associated with poor prognosis (3). In mice models with DLBCL, apoptosis in pericytes, one of the important elements of angiogenesis, and a decrease in tumor volume were observed with imatinib treatment targeting PDGFR-β (4). It was observed that a high level of thrombospondin expression in many tumor cell lines inhibits tumor cell angiogenesis and progression (5,6). The aim of this study is to immunohistochemically investigate the relationship between the staining rates of mainly antiangiogenic factor thrombospondin-1, VEGF, and PDGFR-in tissue preparations in patients diagnosed with DLBCL as a result of lymphadenopathy biopsy and their clinical features at the time of diagnosis, response to treatment and prognosis.

MATERIAL AND METHOD

The study was carried out with the permission of Kocaeli University Noninvasive Clinical Ethics Committee (Date: 13.01.2015, Decision No: 1-23). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

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Study Design and Data Collection

Between June 2007 and September 2014, 44 patients with DLBCL diagnosed by lymph node biopsy in the haematology department were included in the study. Patients whose treatment and polyclinic follow-ups were accepted in our department were included in the study. Pathology preparations of 13 patients for whom reactive hyperplasia was found in the lymph node biopsy and no other malignancies were taken as the control group.

The cut-off values of the laboratory parameters used in the study were chosen as the upper limit of their normal values. The term partial remission was defined as a total reduction of more than 50% of the product of the perpendicular dimensions of the measurable lesions, whereas the term complete remission is defined for patients who show complete recovery with no signs of disease in laboratory values and imaging after treatment (7).

Method of Staining

Immunohistochemical VEGF, PDGFR-β, and Thrombospondin stains were performed with the streptovidin-avidin-biotin method. The preparations were evaluated at 40 magnification under light microscopy with a hematologist and a pathologist were scored as 0, 1, 2, and 3 as per the staining ratio of the cells. The sections were deparaffinized by holding them in a 56oC incubator overnight. Deparaffinization was completed by keeping the sections taken in xylene for 15+15 (30) minutes after they were removed from the oven. Then the absolute alcohol was poured first, kept for 15 minutes in the second, and then the same procedure was performed with 96% ethyl alcohol and hydrated. Washed with distilled water for 5 minutes. The slides were placed in a microwave-resistant plastic bowl. 10% citrate buffer solution was prepared and placed on them (10 cc citrate buffer is prepared with 90 cc distilled water). The microwave oven was operated at maximum power (100%) for 10 minutes. At the end of the time, the power of the oven was reduced by 50% and operated for 5+5 minutes. After the preparations were taken out of the microwave oven, they were kept at room temperature for 20 minutes. They were washed with distilled water. The sections were kept in a mixture of 3% HO (Hydrogen peroxide) for 20 minutes to perform peroxidase blockage. Then the sections were washed with distilled water. After sections were kept in Phosphate buffer saline (PBS) for 15 minutes, they were placed in an immunostaining container and protein blockade was performed for 15 minutes. After washing, VEGF, PDGFR, and Thrombospondin stains were dropped and left for 2 hours incubation. After the preparations were taken into PBS and shaken, they were kept in PBS for 15 minutes for the second time. Goat-Anti-Polyvalent was dropped on the sections taken into the staining container and left for 20 minutes. Then, after the preparations were taken into PBS and shaken again, they were kept in PBS for 15 minutes for the second time. Streptovidin peroxidase was dropped on the sections taken into the staining container and left for 20 minutes. Tissues that were passed through PBS again were incubated with AEC chromogen for 20 minutes. The colored preparations were washed in distilled water and then kept in Mayer hematoxylin for 2 minutes. After washing with distilled water, soaked through ammonia water. After the sections were washed with distilled water again and dried, they were sealed with a suitable covering medium. The preparations evaluated at 40 magnification under light microscopy with a hematologist and a pathologist were scored as 0, 1, 2, and 3 as per the staining ratio of the cells.

Statistical Analyses

The evaluation of the results was made using the SPSS Version.22.0 program. Variables in the study were evaluated in terms of normal distribution using the One-Sample Kolmogorov-Smirnov test. Data that are in compliance with the normal distribution were given with arithmetic mean and standard deviations, and data that are not in compliance with the normal distribution were given with median values (25% -75% percent). Chi-square and Fisher tests were used for comparison of categorical data. Non-parametric Mann-Whitney test was used to evaluate ordinal data. Kaplan-Meier method and Log-rank test were used to calculate progression-free survival (PFS) and overall survival times (OS). Progression-free survival time was considered as the time from diagnosis to progression, and total survival time from the date of diagnosis to the date of patient death or the termination of follow-up period. $P<0.05$ values were considered statistically significant.

RESULTS

A total of 44 cases with a diagnosis of DLBCL, 24 male, 20 female, diagnosed as a result of lymph node biopsy in Kocaeli University Medical Faculty Hospital, Hematology Department between June 2007 and September 2014 and 6 male and 7 female, totally 13 lymphadenopathy pathology preparations compatible with reactive hyperplasia were taken as the control group.

The mean age was found to be 60.55±11.23 years (median 60 years) in the patient group and 42.53±14.52 years (median 44 years) in the control group. **Table 1** presents clinical and laboratory characteristics of the patient and control groups. When the patients were evaluated as per the IPI score, it was observed that 31.8% were low risk, 27.3% low-intermediate risk, 27.3% high-intermediate risk, and 13.6% high risk. When we examine the treatment protocols given

to the patients in primary care, 35 patients (79.5%) R-CHOP (Rituximab, Cyclophosphamide, Adriamycin, Vincristine, Prednisolone), 2 patients (4.5%) RCVP (Rituximab, cyclophosphamide, vincristine, methylprednisolone), 2 patients (4.5%) CHOP (Cyclophosphamide, adriamycin, oncovin, prednisone), 1 patient (2.3%) CVP (cyclophosphamide, vincristine, methylprednisolone), 1 patient (2.3%) received cyclophosphamide and methylprednisolone. VEGF, PDGFR- and thrombospondin staining rates were depicted in **Table 2.** PDGFR-β staining was statistically significantly lower in the patient group compared to the control group (p=0.009). In terms of staining with thrombospondin-1, a low rate of staining was found in 41 (93.2%) of the patients and a high rate of staining in 3 patients (6.8%). In the control group, low staining was detected in 12 patients (92.3%) and high staining in 1 patient (7.7%). There was no statistically significant difference was found between the two groups in terms of thrombospondin-1 staining rates.

Table 2. Comparison of patient and control groups according to thrombospondin-1, VEGF, PDGFR-β staining rates

	Total n (%)	Control n (%)	Patient n (%)	p
VEGF				0.742
0-1	38 (66.7)	8 (61.5)	30 (68.2)	
2-3	19 (33.3)	5 (38.5)	14 (31.8)	
PDGFR-β				0.009
0-1	36 (63.2)	4 (30.8)	32 (72.7)	
2-3	21 (36.8)	9 (69.2)	12 (27.3)	
Trombospondin-1				1.00
0-1	53 (93)	12 (92.3)	41 (93.2)	
2-3	4 (7)	1 (7.7)	3 (6.8)	

When the statistical relationship between VEGF, PDGFR-β, and Thrombospondin was investigated, a significant relationship was found between PDGFR-β and Thrombospondin (p=0.003, r=-0.440). **Table 3** presented a comparison of the general characteristics of the patients and the staining rates of VEGF, PDGFR-B, and Thrombospondin-1. It was observed that as the PDGFR-β staining rate increased, it was observed the thrombospondin-1 staining rate decreased. VEGF was found to be stained at a statistically significant higher rate in men than in women (p=0.029). When the VEGF, PDGFR-β, thrombospondin-1 staining ratios of the patients and IPI score, whether they have B symptoms, disease stage, bone marrow involvement, hepatomegaly, splenomegaly, bulky disease, extranodal involvement, refractory to primary care and relapse were compared, no statistically significant difference was found. It was observed that PDGFR-β was less stained in patients with high IPI scores and stages. Although those with a high PDGFR-β staining rate were mostly female, the rate of males was higher in low staining rates. Less bone marrow involvement, hepatomegaly, splenomegaly, bulky disease, extranodal involvement, and relapse were found in patients with high PDGFR-β staining. However, no statistically significant relationship was observed. Although most of the thrombospondin-1 stained ones were male, all of those highly stained were male. All patients with high thrombospondin-1 staining had B symptoms, and none of them had bone marrow involvement, hepatomegaly, bulky disease, extranodal involvement, refractoriness to treatment, or relapse.

Table 1. Clinical characteristics of the patients and the control group

	Patients	Control group	p
Number (%)	44 (77.1%)	13 (22.9)	
Age (Median (min-max))	60.5 (35-88)	42.53 (16-67)	<0.001
Gender (Male) (%)	24/20 (54.5)	6/7 (46.2)	0.594
LDH elevation (%) (LDH>220 U/L)	35 (79.5)	2 (15.4)	
B symptom positivity (%)	19 (43.2)		
ECOG ½ (%)	38/6 (86.4/ 13.6)		
Stage (n)	8/13/14/9		
I/II/III/IV (%)	(13.8/ 29.5/ 31.8/ 20.5)		
IPI (n)	4/10/12/12/6		
(0/1/2/3/4) (%)	(9.1/ 22.7/ 27.3/ 27.3/ 13.6)		
Hepatomegaly (%)	6 (13.6)		
Splenomegaly (%)	11 (25)		
Bone marrow infiltration (%)	4 (9.1)		
Extra-nodal involvement (%)	8 (18.2)		
Bulky disaese (%)	5 (11.4)		
Partial remission (%)	4 (9.1)		
Complete remission (%)	30 (68.2)		
Relapse (%)	5 (11.4)		
Mortality (%)	11 (25)		
Treatment steps taken (n)	38/4/2		
(I/II/III) (%)	(86.4/ 9.1 /4.5)		

Table 3. Comparison of the general characteristics of the patients and the staining rates of VEGF, PDGFR-B and Thrombospondin-1

	VEGF (0-1) n (%)	VEGF (2-3) n (%)	p	PDGFR (0-1) n (%)	PDGFR (2-3) n (%)	p	Thrombospondin (0-1) n (%)	Thrombospondin (2-3) n (%)	p
Gender			0.029			0.477			0.377
Female	17 (56.7)	3 (21.4)		13 (40.6)	7 (58.3)		20 (48.8)	0 (0)	
Male	13 (43.3)	11 (78.6)		19 (59.4)	5 (41.7)		21 (51.2)	3 (100)	
IPI			0.419			0.332			0,558
<3	16 (53.3)	10 (71.4)		17 (53.1)	9 (75)		25 (61)	1(33,3)	
≥3	14 (46.7)	4 (28.6)		15 (46.9)	3 (25)		16 (39)	2(66,7)	
B symptoms in diagnosis	17 (56.7)	8 (57.1)	1.00	19 (59.4)	6 (50)	0.828	22 (53,7)	3 (100)	0,247
Stage			0.596						0.599
≤2	13 (43,3)	8 (57.1)		14 (43.7)	7 (58.3)	0,601	19 (46.3)	2 (66.7)	
>2	17 (56,7)	6 (42.9)		18 (56.3)	5 (41.7)		22 (53.7)	1 (33.3)	
Bone marrow involvement in diagnosis	3 (10)	1 (7.1)	1.00	3 (9.4)	1 (8.3)	1.000	4 (9.8)	0 (0)	1.000
Hepatomegaly in diagnosis	6 (20)	0 (0)	0,155	5 (15.6)	1 (8.3)	1.000	6 (14.6)	0 (0)	1.000
Splenomegaly in diagnosis	8 (26.7)	3 (21.4)	1.000	7 (21.9)	4 (33.3)	0.457	10 (24.4)	1 (33.3)	1.000
Bulky disease in diagnosis	2 (6.7)	3 (21.4)	0,307	4 (12.5)	1 (8.3)	1.000	5 (12.2)	0 (0)	1.000
Extranodal involvement in diagnosis	7 (23.3)	1 (7.1)	0.402	7(21.9)	1 (8.3)	0.403	8 (19.5)	0 (0)	1.000
Refractory disease	3 (10)	1 (8.3)	1.000	4 (12.9)	0 (0)	0.563	4 (10)	0 (0)	1.000
Relapse on follow-up	3 (10)	2 (15.4)	0,630	3 (9.7)	2 (16.7)	0.608	5 (12,5)	0 (0)	1.000

Considering in terms of VEGF staining rates, the estimated 5-year OS in low-stained and high-stained patients was 71% in both groups. While the estimated 5-year PFS value was found to be 63% in low-stained patients, it was 73% in high-stained patients. Although it was not statistically significant in those highly stained with PDGFR-β, a decrease in both OS and PFS values was observed in the 5-year estimate. In the PDGFR-β low staining group, the estimated 5-year OS was 72% PFS was 69%, while the OS was found as 70% and PFS was 45% in the high staining group. The 5-year OS was found to be 66% in the thrombospondin-1 high-staining group, compared to 72% in the low-stained patients (**Table 4**).

Table 4. Comparison of VEGF, PDGFR-β, Thrombospondin-1 staining rates and overall and progression-free survival of patients

	5-year OS (%)	p	5-year PFS (%)	p
VEGF		0.689		0.473
Low (<2)	71%		63%	
High (≥2)	71 %		71%	
PDGFR		0.773		0.276
Low (<2)	72 (%)		69 (%)	
High (≥2)	70 (%)		45 (%)	
Thrombospondin		0.500		0.679
Low (<2)	72		64	
High (≥2)	66		66	

DISCUSSION

DLCHL is the most common subtype of NHL in adults and constitutes approximately 40% of cases. It has been observed that the prognosis is worse and the long-term survival is less in patients who do not fully respond to the initial treatment. Therefore, studies have been conducted to investigate more effective and less toxic chemotherapy drugs and biological agents for this specific and large patient group. Among the treatment targets of these studies, angiogenesis, which is of increasing importance and the main subject of many cancer treatment studies, is included (8). Our aim in this study is to immunohistochemically study the relationship between the staining rates of mainly antiangiogenic factor thrombospondin-1, angiogenic VEGF, and PDGFR-in tissue preparations in patients diagnosed with DLBCL as a result of lymphadenopathy biopsy and their clinical features at the time of diagnosis, response to treatment and prognosis.

In the present study, it was observed that most of the patients were stained with VEGF, although PDGFR-β was lower in patient group. We found a statistically significant negative correlation between thrombospondin-1 and PDGFR-β staining. Over-staining of VEGF and under-staining of thrombospondin-1 shows us that the balance in lymphoma favors angiogenetic factors.. In addition, the fact that PDGFR β staining rate is prominent while thrombospondin-1 staining rate is low supports this. In another study by Paydas et al. (9), TSP-1 expression rate was found to be 14.8% in the pathological preparations of 88 patients with DLBCL, and no relation with prognostic factors and survival could be demonstrated.

When the literature is reviewed, there are studies showing the relationship between serum VEGF level and progression and prognosis of cancers. VEGF expression in aggressive lymphoma subtypes such as DLBCL, peripheral T-cell lymphoma, mantle cell lymphoma, and primary effusion lymphoma have been shown to

be highly and slightly increased in chronic lymphocytic leukemia/small lymphocytic leukemia (CLL/SLL) (10-12). Especially in acute lymphocytic leukemia and lymphomas, it was found that some tumor cells expressing VEGFR-1 and VEGFR-2 are involved in the survival, metastasis, and proliferation of tumor cells by autocrine mechanisms (13,14). The current study revealed that VEGF was found to be low in those with low IPI scores, and higher staining rates in patient group. Contrary to this extranodal involvement, hepatomegaly, bulky disease, splenomegaly, bone marrow involvement, refractory, and relapse rates were found to be high in patients with low VEGF staining. In the study conducted by Riihijarvi et al. (15). in 102 high-risk patients under 65 years of age, it was found that serum VEGF levels were statistically high in patients with high IPI scores, and performance score and no relationship was found between serum VEGF level and gender, B symptom, bulky disease. Again, in the study conducted by Salven et al. on 200 patients with NHL, no significant relationship was found between serum VEGF levels and stage, bulky disease, presence of B symptoms, extranodal involvement, and histological grade, but a significant relationship was found between performance score (ECOG) and IPI (16).

In another study, Hazar et al. demonstrated that VEGF negative NHL patients had better treatment responses than VEGF positive NHL patients (17). In the study of Salven et al., it was shown that serum VEGF level is an independent indicator of poor prognosis for NHL patients. In a study conducted on 200 NHL patients, high pre-treatment serum VEGF levels (462 pg ml 7-1) were found to be associated with high LDH levels, low-performance status, and low survival. The 5-year survival rate was found to be 31% in those with high serum VEGF levels, it was found 61% in those with low serum VEGF levels ($p<0.001$). The 5-year survival was found as 30% in patients with high VEGF serum levels and 53% in patients with low VEGF ($p <0.001$) in patients with DLBCL (n=78) (16). In our study, no significant relationship was found between VEGF expression at the tissue level and overall survival and progression-free survival.

Although the prognostic value of VEGF expression at the tissue level in patients with DLBCL is uncertain, it has been associated with poor prognosis in studies with serum VEGF levels (3,18). We thought that one of the reasons for its uncertain expression at the tissue level may be due to the fact that VEGF interacts with many inflammatory processes, and there are many factors affecting its expression at the tissue level.

When PDGFR-β staining rates were compared with demographic data, it was observed that patients with higher IPI scores and stages had less staining. When we looked at the survival analysis, we noticed that the group with a high PDGFR-β staining rate tended to have lower both OS and PFS values. Studies have also found conflicting results regarding angiogenic factors related to NHL depending on the heterogeneous population, tissue sample, or serum sample taken. Agreeably it was found that the rate of PDGFR-β staining was higher in the control group. It can be considered that this contradictory result may be influenced by the angiogenetic environment in reactive lymphadenopathy in the control group There are not many studies on the relationship between PDGFR-β expression in tissue preparations and prognosis in patients with DLBCL. As a result of its ligand binding to PDGFR-β, the PDGF receptor, the tyrosine kinase pathway is activated, which induces cell proliferation, differentiation, and migration. Tyrosine kinase inhibitors such as imatinib and sunitinib targeting PDGFR – β have been shown to be effective in some solid tumors by decreasing pericyte density around the vessel and weakening angiogenesis (19,20). In neonatal mouse models in which PDGFR was functional blockade, it was observed that a number of vascular smooth muscle cells were inhibited, apoptosis of vascular endothelial cells was induced and glomerular vascular network formation was negatively affected (21). In the study of Ruan et al. which they performed with imatinib targeting PDGFR -β in mouse lymphomas, they have demonstrated antiangiogenic effects in pericytes. It was observed that in mice with 3 types of DLBCL models, with 2-3 weeks of imatinib treatment, PDGFR -β + pericytes apoptosis and a significant decrease in tumor volume were observed. It was thought that the decrease in pericytes expressing PDGFR-β was due to increased apoptosis of CD 31+ vascular endothelial cells and decreased tumor vascularity (4).

TSP-1 is a multifunctional protein found in many biological processes such as angiogenesis, apoptosis, TGF-beta activation, and immune regulation. In some studies, thrombospondin has been shown to be a negative regulator of tumor progression and angiogenesis. In the current study, it was observed that the 5-year OS value in the group with a high level of thrombospondin-1 staining was lower than the patients with a low level of staining, although it was not statistically significant. It was observed that a high level of thrombospondin expression in tumor cell lines such as breast, skin, colorectal, glioblastome, and hemangioblastoma inhibits tumor cell angiogenesis and progression (5,6). On the other hand, thrombospondin is found as an adhesive protein in the extracellular matrix in many epithelial cancers and has been shown to be effective in cancer progression. Because thrombospondin-1 has been shown to activate the plasminogen/plasmin system in many adenocarcinoma models and increase tumor progression and metastasis (22). In the study of

Paydas et al. (23) on tissue preparations of 177 NHL patients, it was found that thrombospondin expression was associated with aggressive morphology. In addition, patients expressing both thrombospondin and survivin were shown to have both aggressive morphology and shorter OS. Furthermore, no relation was found between B symptoms, extranodal involvement, hepatomegaly, splenomegaly, and performance score used in daily practice.

This study has some limitations. The patients used as a control group in our study underwent lymph node excisional biopsy with suspicion of lymphoma and were evaluated as reactive lymph nodes on pathological examination. It should be kept in mind that factors such as viral infection that may cause reactive lymph nodes may impair the angiogenetic expression profile.

CONCLUSION

In this study, it was observed that the rate of VEGF staining was higher in patients with anemia, leukopenia, and lymphopenia with prognostic importance. Although it was not statistically significant 5-year OS and PFS values were low in patients with high levels of expression PDGFR-β and thrombospondin-1. However, another conclusion to be drawn from our study is that angiogenetic factor expression should not be used in the differentiation of reactive/tumoural lymph node enlargement. Special agents specific to the person and the type of DLBCL may be developed in the future with studies that target VEGF, PDGFR-β, and thrombospondin-1, with genetic studies and examining multiple immunohistochemical markers, in which there is a larger patient and control group for treatment in patients with DBBHL.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Kocaeli University Noninvasive Clinical Ethics Committee (Date: 13.01.2015, Decision No: 1-23).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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