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Kronik Lenfositik Lösemi Tanılı Hastalarda Vasküler Endotel Büyüme Faktör (VEGF) Düzeylerinin Prognozla İlişkisi

The Association Between Vascular Endothelial Growth Factor (VEGF) Levels And Prognosis In Patients Chronic Lymphocytic Leukemia

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ÖZ

GİRİŞ ve AMAÇ: *Rai ve Binet evreleme sistemleri, kronik lenfositik lösemi(KLL)'nin prognozunu değerlendirmek için standart bir yöntem olarak kullanılırlar. Bununla birlikte, KLL klinik özellikler ve prognoz açısından heterojen bir hastalıktır. Rai ve Binet evreleme sistemleri; sağkalım sonuçlarını ve tedaviye cevabı etkileyen KLL hücrelerinin farklı biyolojik özelliklerini yansıtmayabilir. Bu nedenle, son yıllarda yeni prognostik parametreler önemli hale gelmiştir. KLL'deki serum VEGF düzeylerinin, hastalığın ilerlemesini belirlemede önemli bir parametre olabileceği önerilmektedir.*

YÖNTEM ve GEREÇLER: *46 KLL hastasında ve 20 sağlıklı kontrolde; serum VEGF düzeylerini ELISA yöntemi ile tespit ettik.*

BULGULAR: *KLL hasta grubunda; sağlıklı kontrol grubuna göre daha yüksek ortalama serum VEGF düzeyleri saptandı (sırasıyla; 267 pg / mL, 43,8 pg/mL, p: <0,0001). Daha yüksek ortalama serum VEGF düzeyleri (> 267 pg / ml), Binet A ve B KLL hastalarında daha kısa hastalık progresyonu süresi ile ilişkiliydi (p: 0,004). Ayrıca yüksek serum VEGF düzeylerinin OS ile anlamlı korelasyon gösterdiğini saptadık (p: 0,019).*

TARTIŞMA ve SONUÇ: *Sonuç olarak, KLL hastalarındaki serum VEGF düzeylerinin kontrol grubundan daha yüksek olduğunu ve hastalığın ilerlemesi ve sağkalımı ile ilişkili bir parametre olabileceğini bulduk. Bu nedenle; ELISA ile serum VEGF kolay tespit edilmesi, daha büyük ve çok değişkenli çalışmalarda önemi gösterildikten sonra, yararlı bir klinik parametre olabilir.*

Anahtar Kelimeler: *Kronik Lenfositik Lösemi, Vasküler Endotel Büyüme Faktörü, Sağkalım*

ABSTRACT

INTRODUCTION: *The Rai and Binet staging systems are used as a standard method to evaluate the prognosis of chronic lympocytic leukemia(CLL). However, CLL is a heterogeneous disease in terms of clinical features and prognosis. The Rai and Binet staging systems; may not reflect the different biological properties of CLL cells affecting survival outcomes and response to treatment. Because of this, new prognostic parameters has become important in the recent years. It is suggested that serum VEGF levels in CLL may be an important parameter in determining the progression of the disease.*

METHODS: *We detected the serum levels of VEGF in 46 CLL patients and 20 healthy controls with the use of ELİSA method.*

RESULTS: *The CLL patients group had higher median serum levels of VEGF compared to the control group (267 pg / mL, 43,8 pg/mL, p: <0,0001, respectively). Higher median value of serum VEGF levels (>267 pg/ml) were associated with a shorter time of disease progression in Binet A and B CLL patients (p: 0,004). We also found that high serum VEGF levels showed a significant correlation with OS (p: 0,019).*

DISCUSSION AND CONCLUSION: *As a result, we found that the serum levels of VEGF in the CLL patients are higher than the control group and it can be a parameter associated with the disease progression and survival. For this reason and because the simplicity of analyzing with ELISA, it can be a useful clinical parameter, after its importance have been shown in larger and multi-variate studies.*

Keywords: *Chronic Lymphocytic Leukemia, Vascular Endothelial Growth Factor, Survival*

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INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is characterized by the increase in mature malignant B- lymphocytes in the peripheral blood, bone marrow, lymph nodes and secondary lymph tissues, such as the spleen, liver; and is the most common type of adult leukemia in Western countries(1,2). The most of patients with CLL have early-stage disease at the time of diagnosis. A significant number of these patients do not require any treatment during their follow-up and have the same life expectancy in the age and gender-matched general population. The other patient population has a rapidly aggressive clinical course, needs treatment during diagnosis and has poorer survival outcomes (3).

There are two staging systems for CLL. These are the Binet staging system and the Rai staging system(4,5). Both of these staging systems, a significant relationship are observed between the stage and life expectancy. The patients who were diagnosed in the early stage live longer. CLL is a B-cell malignancy that still remains incurable. In early-stage or asymptomatic CLL , treatment has not been proven to improve survival; thus, the majority of patients are monitored through a "watch and wait" approach to determine progression of the disease and develop the symptoms (6,7). However, several studies conducted in recent years have shown that the disease course is not homogeneous in patients with CLL and that the staging systems of Binet or Rai may not be applied to each patient in the same way in evaluating the prognosis. Because some patients with early stage disease may progress rapidly. The Rai and Binet staging systems; may not reflect the different biological properties of CLL cells affecting survival outcomes and response to treatment (8,9).

Apart from the Binet or Rai staging systems, many new prognostic parameters have been defined in CLL. New prognostic markers such as lymphocyte doubling time(LDT), lactate dehidrogensa (LDH), beta-2 microglobulin (B-2 M), thymidin kinase, immunoglobulin heavy-chain variable region gene (IGHV) mutation status, FISH cytogenetics, and CD38 and ZAP-70 (zeta-associated protein) expression are described(3,9-11). Recently, angiogenesis has been reported to play a role in the pathogenesis of CLL(12-16). Vascular endothelial growth factor (VEGF) plays an important role in the formation and activation of angiogenesis.VEGF levels can be detected in serum, leukemic cells and bone marrow in CLL patients. Serum VEGF levels can be easily determined by Enzyme Linked Immunosorbent Assay (ELISA). There are studies suggesting that increased serum VEGF levels correlate with disease progression in CLL (14-16). Therefore, we aimed to investigate the relationship between serum VEGF levels and disease prognosis in patients with CLL who were followed in the Department of Hematology, Cerrahpaşa Medical Faculty .

MATERIALS AND METHODS

1.Patient Outcomes

Fourty-six CLL patients who were newly or previously diagnosed and treated in Istanbul University Cerrahpaşa Medical Faculty Hematology Department and 20 healthy controls were enrolled to this study. The healthy control group consisted of 20 person , including 13 male and 7 female patients with no known to cause diseases (collagenosis, psoriasis, diabetes and chronic liver disease). All the control subjects were matched with the population of the patients' group in terms of age and sex. The study was conducted according to good clinical and laboratory practice rules and the principles of the Declaration of Helsinki, and approved by the local ethics committee. After written consent was obtained from all patients and healthy controls before enrollment into the study , the blood sample was taken. The patients were diagnosed CLL according to the National Cancer Institute Working Group (NCIWG) (1).

Data on sex, age, complete blood count, peripheral smear and laboratory results including LDH and B-2 M; immunophenotypingof peripheral blood and bone marrow finding were registered at the enrollement time of this study in CLL patients . Moreover, data about disease

characteristics and treatment protocols of CLL patients were also collected.

The levels of peripheral blood leukocyte and lymphocyte counts were designated as 50.000/mm³ Fourty-two of 46 patients (91%), the rate of CD38 expression was determined by flow cytometry.Patients were grouped according to CD38 positivity with two different cut-off levels in which were 20% and 30% low or elevated. Six out of 46 patients couldn't perform bone marrow examination.The remaining patients (87%) were grouped according to the diffuse and nondiffuse according to the criteria defined by Rozman (17). LDH(U/L) and B-2 M (mg/dl) levels were designated normal or elevated according to our laboratory cut-off values (upper normal limit). The patients were grouped the LDT in less or higher than 12 months.

The median follow-up time from the detection of VEGF level was 13 months (range 11-16 months. In our study, we used progression-free survival as the endpoint. We defined the disease progression as the change to a more advanced clinical stage in the Binet staging system. While evaluating the disease progression, the patients in the Binet C stage were excluded from the study because they did not have the opportunity to move to a more advanced clinical stage. Therefore, disease progression was evaluated in a total of 35 patients in the Binet A and B stage.

2.Determination of Serum VEGF Level

Blood samples were collected from the patients, and the controls and the sera were stored at -80 ◦C. Serum VEGF level were measured using a commercially available sandwich ELISA kits (The Biosource International Human VEGF Immunoassay) according to the manufacturer's instructions. Results were reported in picograms per milliliter (pg/mL).

STATISTICAL ANALYSIS

Statistical analyses were performed with SPSS (SPSS Inc, IBM, USA).The variables were not normally distributed, non-parametric methods were used for analyses. The Mann Whitney U test was utilized to compare non-parametric variables between the two groups. The correlation coefficients between different parameters was calculated by using the Spearman correlationand the Chi-square test. Survival durations were calculated via the Kaplan-Meier method. The log-rank test was employed to compare cumulative survival in the patient groups. All statistical analysis were two sided and significance was defined as $P < 0.05$.

RESULTS

Patient Outcomes

A total of 46 patients (15 female, 31 male; median age 62 years) and 20 HC (7 female, 13 male; median age 65 years) were enrolled to this study. At the time of diagnosis, 25 patients (54%) were classified as Binet A, 10 patients (22%) were classified as Binet B and 11 patients were classified as Binet C (24%). According to the Rai staging system, 9 patients (20%) were Rai 0; 13 patients (28%) were Rai I; 12 patients (26%) were Rai II; Five patients (11%) were Rai III; 7 patients (15%) were in Rai IV stage. Serum VEGF levels were evaluated in 6 patients(13%) at the time of diagnosis and in 40 patients (87%) during the course of the disease. At the beginning of the study, 20 patients (43%) were receiving treatment for CLL. Three patients who were treated were Binet A, 6 patients were Binet B and 11 patients were Binet C. Patients' clinical characteristics are shown in Table 1 .

Table 1: Clinical Characteristics of CLL patients

	N	%
Age		
<65	21	46
≥65	25	54
Gender		
Female	15	33
Male	31	67
Rai Stage		
0	9	20
I	13	28
II	12	26
III	5	11
IV	7	15
Binet Stage		
A	25	54
B	10	22
C	11	24
At the beginning of the study		
Under treatment	20	43
No treatment	26	57

Serum VEGF Level and Correlation with Cinical Features

The serum VEGF level in CLL patients (267 pg/mL (range 73-937 pg/ml) was significantly higher than serum VEGF level (43,8 pg/mL; range:4,5-160 pg/ml) in the HC (p:<0,0001).

Serum VEGF level of patients with peripheral blood leukocyte and lymphocyte counts > 50.000 mm3 were significantly increased when compared to patients with peripheral blood leukocyte and lymphocyte counts ≤ 50.000 mm3 (p<0,001 respectively).

Serum VEGF level of patients with Binet B,C and Rai II,III ,IV were significantly increased when compared to patients with Binet A and Rai 0,I(p<0,038; p<0,027 respectively). Additionally, serum VEGF level was significantly elevated in the patients who have diffuse bone marrow infiltration higher than non-diffuse bone marrow infiltration (p<0,007).However,there was no association between serum VEGF level and age, gender, hb level, platelet count, LDT,the rate of CD38 expression (20% and 30%), LDH and B-2 M (Table 2) .

Survival Analysis

In the Binet A and B CLL stage, the factors affecting the disease progression could not be evaluated in multivariate analysis because of the insufficient number of cases, only univariate analyzes were performed. Eleven of the 35 patients in the Binet A and B stage had disease progression. 5 patients (46%) from Binet A to B; 2 patients (18%) from Binet A to C; four patients (36%) from Binet B to C progressed. Ten patients with serum VEGF levels elevated the median value (> 267 pg / ml) had stage progression. PFS was median 18 months (12-60) from the diagnosis and 9 months during the study period. Only one patient with serum VEGF level less the median value(≤267 pg/ml) had stage progression and there was no median time in this group Progression free survival (PFS) of patients with high serum VEGF level (>267 pg/mL) was significantly decreased in comparison to patients with low serum VEGF level (≤267 g/mL)(p=0,004) **(Figure 1).**

Table 2 : Serum VEGF Level and Correlation with Cinical Features

Characteristics	Median VEGF	p value
Age		
≤ 65	304,22	
> 65	245,98	**0,406**
Gender		
Male	283,10	
Female	270,22	**0,386**
Leucocyte Count		
≤ 50.000/mm³	229,62	
> 50.000/mm³	342,97	**0,001**
Lymphocyte Count		
≤ 50.000/mm³	236,10	
> 50.000/mm³	351,90	**0,001**
Hb Level		
≤ 12 gr/dl	274,14	
> 12 gr/dl	285,08	**0,231**
Platelet Count		
≤ 150.000/mm³	274,62	
> 150.000/mm³	291,01	**0,812**
LDT		
> 12 month	265,63	
≤ 12 month	328,47	**0,352**
Bone marrow involvement		
Nondiffuse	203,67	
Diffuse	360,07	**0,07**
CD 38		
≤ % 20	254,47	
> % 20	328,95	**0,076**
CD 38		
≤ % 30	265,73	
> % 30	336,83	**0,224**
LDH		
≤ upper limit	274,92	
> upper limit	**287,12**	**0,314**

Table 2 (continued) : Serum VEGF Level and Correlation with Cinical Features

	Median VEGF	p Value
B-2 M (mg/L)		
≤ 2	276,80	
> 2	279,48	**0,393**
Rai		
0-I.	224,35	
II-III-IV.	328,90	**0,038**
Binet		
A	225,33	
B and C	342,67	**0,027**

VEGF, Vascular Endothelial Growth Factor ; LDT, *lymphocyte doubling time;* B-2 M, Beta-2 microglobulin; LDH, lactate dehydrogenase

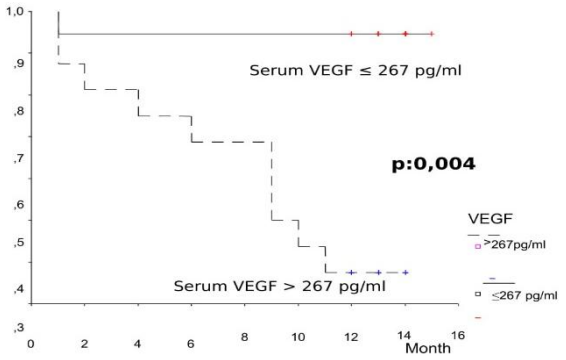

Figure 1. *PFS analysis in Binet A-B patients. Serum high VEGF level 267 pgmL was associated with shorter PFS (p=0,004).*

In the disease progression, PK high leukocyte and lymphocyte counts, LDT less than 12 months, diffuse bone marrow involvement , high B-2 M level, high serum VEGF level were found to be significant. **(Table 3).** We used median VEGF level in MM patients to determine a cut-off value for VEGF level; the cut-off value was 267 pg/mL. Five patients (11%) died at a median of 13 months (range 11–16 months). Two of 5 patients (40%) were in Binet B and 3 patients (60%) were in the Binet C stage.

According to the Rai staging system, one patient (20%) was Rai II; Two patients (40%) were Rai III and 2 patients (40%) were in Rai IV stage). All 5 patients died due to disease activation during the course of CLL. Serum VEGF levels were higher the median level (> 267 pg / ml) in all patients who died (Figure 2). Except for age, sex, Hb level, bone marrow involvement,LDH level, all parameters showed a significant correlation with OS using univariate analysis . These parameters significant on univariate analysis available: higher levels of peripheral blood leukocyte and lymphocyte counts (respectively p:0,007, p:0,001); low platelet count (p:0,004); LDT less than 12 months (p:0,017); the rate of CD38 expression >30% (p:0,008) ; higher B-2 M level (>3,3 mg/L) (p:0,014); advantage Binet ve Rai stage (respectively p:0,01, p:0,024) and higher serum VEGF level (>267 pg/ml) (p:0,019) **(Table 4).**

DISCUSSION

CLL is a disease with variable clinical course. With the widespread use of automated blood counter device in recent years, the majority of CLL patients are diagnosed at an early stage of disease. Patients diagnosed at an earlier stage are usually not treated at the time of diagnosis. Patients diagnosed at advanced stage have higher tumor mass; they require treatment during diagnosis or within a short period of time. Differences in the clinical presentation of the disease make it difficult to estimate survival time for CLL patients. The Rai and Binet staging systems are used to classify patients with CLL, although both of these systems are not very effective for predicting early disease progression(18,19). Therefore, many new prognosis parameters have been determined apart from the Rai and Binet staging systems (11,12).

Angiogenesis is a physiological process involving the formation and growth of new blood vessels from pre-existing vessels. Angiogenesis, especially in wound healing and pregnancy, plays a role in the growth and development of the body or organ. Recent studies have shown that the importance of angiogenesis may play role in the pathophysiology of hematopoietic cell development and various hematological malignancies.

Table 3: Factors Affecting Disease Progression in Binet A and B CLL

PARAMETER		P value
Age	≤ 65 vs > 65	**0,758**
Gender	M/F	**0,125**
Leucocyte Count	≤50.000/mm³ vs > 50.000/mm³	**0,0001**
Lymphocyte Count	≤50.000/mm³ vs> 50.000/mm³	**0,002**
Hemoglobin Level	≤ 12gr/dl vs > 12gr/dl	**0,728**
Platelet Count	≤150.000/mm³ vs >150.000/mm³	**0,680**
LDT	≤ 12 months vs > 12 months	**0,02**
Bone marrow involvement	Nondiffuse / Diffuse	**0,027**
CD 38 Expression	≤ % 20 vs > % 20	**0,343**
CD 38 Expression	≤ % 30 vs > % 30	**0,361**
LDH	≤ normal vs > normal	**0,296**
B-2 M	≤ normal vs > normal	**0,004**
Serum VEGF	≤ 267 pg/ml vs > 267 pg/ml	**0,004**

VEGF, Vascular Endothelial Growth Factor ; **LDT,** *lymphocyte doubling time;* **B-2 M,** Beta-2 microglobulin; **LDH,** lactate dehydrogenase

Table 4. Factors Affecting Survival in CLL

PARAMETER		P VALUE
Age	≤ 65 vs > 65	**0,137**
Gender	M / F	**0,706**
Leucocyte Count	≤50000/mm³ vs > 50000/mm³	**0,007**
Lymphocyte Count	≤50000/mm³ vs > 50000/mm³	**0,001**
Hemoglobin Level	≤ 12gr/dl vs > 12gr/dl	**0,08**
Platelet Count	≤150000/mm³ vs >150000/mm³	**0,004**
LDT	≤ 12 months vs > 12 months	**0,017**
Bone marrow involvement	Nondiffuse / Diffuse	**0,1**
CD 38 Expression	≤ %20 vs > % 20	**0,120**
CD 38 Expression	≤ % 30 vs > % 30	**0,008**
LDH	≤ normal vs > normal	**0,735**
B-2 M	≤ nornal vs > normal	**0,014**
Rai Stage	0-1 vs 2-3-4	**0,024**
Binet Stage	A vs B-C	**0,01**
Serum VEGF	≤ 267 pg/ml vs > 267 pg/ml	**0,019**

VEGF, Vascular Endothelial Growth Factor; **LDT,** *lymphocyte doubling time;* **B-2 M,** Beta-2 microglobulin; **LDH**, lactate dehydrogenase.

Cell surface receptors and various stimulatory and inhibitory angiogenic factors mediate and balance the complex process of angiogenesis .The main cytokines inducing angiogenesis is VEGF and basic-fibroblast growth factor (b-FGF) (12,20) VEGF is one of the most important proangiogenic factors in angiogenesis. In addition to its role in angiogenesis, VEGF is a known survival factor for various cell types including endothelial cells, hematopoietic stem cells and solid tumor cells (21-22).

There are studies suggesting increased angiogenesis in bone marrow and / or lymph node in CLL (13-16, 23-26). Higher serum or plasma levels of angiogenic factors such as bFGF, VEGF and Angiopoietin-2 have been reported in CLL patients compared to normal controls(14-15, 26-30)

Serum or plasma samples were used to determine the level of VEGF. Some studies have shown that serum VEGF levels are higher than plasma VEGF levels (30) Molica et al.investigated that whether serum and plasma VEGF levels were different with the concern that they might be affected by the presence of platelets. Molica et al. showed that this was not different in patients with CLL. (14.).

We evaluated serum VEGF level by ELISA method as in other studies in the literature. (14-15).

In our study, we found that while serum VEGFlevels were higher in CLL patients compared to HC

Molica et al. described that serum VEGF level in CLL patients were higher than HC similar to our study. (14-15). Another study showed that plasma VEGF levels were also higher in CLL patients compared to the control group (30). Basic fibroblast growth factor (bFGF) and VEGF are elevated in peripheral blood plasma of patients with chronic lymphocytic leukemia and decrease after intensive fludarabine-based treatment (27).

In our study, serum VEGF levels of patients advanced Rai or Binet stge (Rai II, III, IV or Binet B,C) , higher leucocyte or lymphocyte counts , and diffuse bone marrow involvement were significantly elevated. In our study, we could not find a significant relationship between serum VEGF level and LDH, LDT, B-2 M and the rate of CD38 expression. But, serum VEGF levels were higher in patients with shorter LDT (<12 months) but the difference was not significant. Similarly, higher serum VEGF levels were found in those with higher B-2 M levels, but the difference was not significant.We think that this could be due to the majority of our patients who are in the early stage and the small number of patients enrolled to this study.

In our study, our aim is not to examine the known prognosis parameters.But, as expected, high leukocyte and lymphocyte counts, LDT shorter than 12 months, high B-2 M level, diffuse involvement of bone marrow and serum VEGF level were found to be associated with disease progression. However, we think that the demonstration that serum VEGF levels are moving with some of these parameters is an indirect indicator that VEGF may be prognostic parameter. Although we could not perform a multivariate analysis due to the small number of patients, there was a significant relationship between serum VEGF levels and disease progression in the Binet A and B CLL patient groups.

In our study, the presence of high leukocyte and lymphocyte count, low platelet count, shorter than 12 months, CD 38 positivity rate above 30%, high B-2 M level, advanced stage compared to Binet and Rai system and high serum VEGF level was found to be associated withoverall survival . It is significant that serum VEGF levels are similar to other prognosis parameters.

There are aspects that are missing in our study and are open to criticism. The small number of patients and the fact that VEGF levels at the time of diagnosis could not be assessed in all patients may have affected our outcomes. It is another lacking that patients are followed for a relatively short period of timeTherefore, our survival outcomes were limited to univariate analyzes, and the low number of cases did not allow for multivariate analyzes.

CONCLUSION

We found that elevated serum VEGF level is associated with aggressive disease characteristics and moreover CLL patients with elevated serum VEGF level has low OS. Therefore, serum VEGF may be clinically useful parameter if evaluated with long-term follow-up and multivariate analysis in large case series . Another advantage is that serum VEGF can be easily measured in patients with CLL by ELISA method.

On the other hand, we think that the lack of a standard range of serum VEGF levels may be a challenge for routine clinical use. There is still a need for future studies enrolling more patients in order to clarify the prognostic significance of VEGF.

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