Prognostic Significance of the IgVH Mutation Status and Immunohistochemical Analysis of ZAP70 and CD38 in Bone Marrow Biopsies in Chronic Lymphocytic Leukemia

Kronik Lenfositik Lösemili Hastaların Kemik İliği Biyopsi Örneklerinde IgVH Mutasyon Durumu ile ZAP70 ve CD38'in İmmünohistokimyasal Olarak Analizinin Prognostik Önemi

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ABSTRACT Objective: Chronic lymphocytic leukemia (CLL) is one of the most common leukemia type among adults in the industrialized countries. Due to the nature of CLL, it is important to recognize patients with a more rapid course of disease. The goal of our study was to study ZAP70 and CD38 antibodies along with immunglobulin heavy chain variable region (IgVH) mutation status, which have been associated with rapid progression and aggressive clinical course in CLL, and to correlate the expression of these molecules with patterns of bone marrow infiltration. **Material and Methods:** We included 84 bone marrow biopsy samples into the study to determine ZAP70 and CD38 status using immunohistochemistry. Expression patterns for both antibodies were then correlated with the bone marrow infiltration patterns. We also analyzed IgVH mutations in 20 patients using DNA obtained from paraffin-embedded formalin-fixed bone marrow biopsies. These findings were then correlated with immunohistochemical results. **Results:** We identified a positive correlation between the expression patterns of ZAP70 and CD38, factors that were previously identified as poor prognostic factors ($p<0.001$). However, there was no correlation between these two markers and IgVH mutation status ($p=1.000$ and $p=0.931$). In addition, we showed a statistically significant positive correlation with ZAP70 immunostaining, and the necessity for an early intervention ($p=0.046$). ZAP70 and CD38 expressions were statistically significantly correlated with the diffuse pattern infiltration of bone marrow ($p<0.001$ and $p<0.001$, respectively). **Conclusion:** Despite small number of our patients, the findings of our study suggested that ZAP70 and CD38 expression patterns as well as IgVH mutation status might be helpful to determine the course of the disease, and the risk of progression. Particularly ZAP70 immunopositive patients appear to have a faster disease progression, and may require earlier intervention and a closer follow up.

Key Words: Leukemia, lymphocytic, chronic, B-cell; somatic hypermutation, immunoglobulin; ZAP70 protein, mouse; CD38 protein, mouse

ÖZET Amaç: Kronik lenfositik lösemi (KLL), batı toplumunda erişkinlerde en sık görülen lösemi türüdür. KLL'nin klinik seyrinden dolayı, hızlı seyredecek hastaları önceden belirlemek önemlidir. Bu çalışmada amaç, KLL'de hızlı progresyon ve agresif gidişle ilişkileri çeşitli çalışmalarla belirlenen ZAP70 ve CD38 antikorları ile İmmünglobulin ağır zincir değişken bölgesi (IgVH) mutasyon durumu arasındaki ilişkileri değerlendirmek, ve bunların ekspresyonunun kemik iliği infiltrasyon paternleri ile bağlantısını ortaya koymaktır. **Gereç ve Yöntemler:** Çalışmaya retrospektif olarak alınan 84 olguya ait kemik iliği biyopsi örneklerinin parafin bloklarından elde edilen kesitlere avidin-biotin peroksidaz yöntemiyle ZAP70 ve CD38 antikorları uygulandı. Tümör hücrelerinin her iki antikorla boyanma oranları saptanarak birbirleriyle karşılaştırıldı; her bir antikor ise kendi içinde kemik iliği infiltrasyon paternleri açısından değerlendirildi. Ayrıca 20 hastaya ait parafine gömülü kemik iliği biyopsilerinden izole edilen DNA örneklerinde IgVH mutasyon durumu değerlendirildi, immünohistokimya ile ilişkisi araştırıldı. **Bulgular:** Kötü prognostik faktör oldukları yaygın olarak kabul edilen ZAP70 ve CD38'in boyanma oranları arasında istatistiksel olarak pozitif korelasyon ($p<0,001$) bulundu, bu korelasyon bu iki belirleyici ile IgVH mutasyon durumu arasında saptanmadı (sırasıyla $p=1,000$ ve $p=0,931$). Ayrıca ZAP70 boyanması gösteren olguların istatistiksel olarak anlamlı bir şekilde tanı sonrasında daha erken tedavi gereksinimi olduğu gözlendi ($p=0,046$). Kemik iliği infiltrasyonlarından diffüz paternde ZAP70 ve CD38 ekspresyonunun anlamlı biçimde fazla olduğu saptandı (sırasıyla $p<0,001$ ve $p<0,001$). **Sonuç:** KLL'de ZAP70 ve CD38 ekpresyonlarının kötü prognostik belirleyici olarak rutinde kullanılabileceği, IgVH mutasyon durumunun hastalığın seyri ve progresyon açısından riskini belirlemede yararlı olduğunu göstermek için daha geniş vaka gruplarına ihtiyaç olduğu ve kemik iliği biyopsi örneklerinde diffüz infiltrasyonun kötü prognostik faktör olarak dikkate alınmaya devam edilmesi gerektiği sonucuna varılmıştır. Özellikle ZAP70 pozitif olan hastalarda hastalığın daha hızlı seyredebileceği ve erken tedavi gereksinimleri olabileceği düşünülerek, bu hastalar yakın klinik takip altında tutulmalıdır.

Anahtar Kelimeler: Lösemi, lenfositik, kronik, B-hücreli; somatik hipermutasyon, immünoglobulin; ZAP70 proteini, fare; CD38 proteini, fare

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Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease characterized by proliferation of the mature CD5 and CD19 positive B lymphocytes in blood, bone marrow and lymphoid organs. This leukemia type is one of the most common leukemias in the Western world.[1] It comprises approximately 40% of all leukemias, and is more common in adults.[2,3] The clinical course of CLL is quite variable. Due to this variability, it is important to recognize those with a more rapid disease progression in order to establish effective treatment earlier in such patients.[4]

Numerous factors have been reported as potentially significant parameters to determine the prognosis of CLL.[5,6] Disease stage still appears to be the most important prognostic factor.[7] The stage and scheme by Rai and Binet is quite helpful in determining the therapeutic needs and median survival probabilities of patients with CLL. However, the prognostic value of this classification scheme is limited in the early stages of the disease.[4] Therefore, numerous studies have been conducted to determine the prognostic factors in the early stages of CLL. These studies have investigated a number of molecular, genetic and biological markers as potential prognostic factors.[8,9] Cytogenetic anomalies, mutations in the immunoglobulin heavy chain (IgVH), Zeta chain related protein kinase 70 (ZAP70), and CD38 are biological markers that have been identified as independent factors influencing clinical course and prognosis of these patients.[3,5-7,10,11]

Recent studies identify two different cell groups in CLL. These groups include cells demonstrating IgVH mutation that are presumed to be pre-germinal center in origin (U-CLL), and germinal or postgerminal center cells with somatic mutations (M-CLL). IgVH mutation is seen in approximately 50% of patients with CLL. Patients with IgVH mutation seem to have a more favorable clinical course, require fewer therapeutic interventions, and have longer remission periods. Overall survival probability also seems to be higher in this patient group. Therefore, the presence of IgVH gene mutation underscores a better prognosis in CLL patients.[12-15] Unfortunately, IgVH mutation can only determined in only a few laboratories. Furthermore, the analysis of molecular genotype is prohibitively expensive to allow routine diagnosis and patient management. Therefore, new studies focus on additional prognostic factors and gene mutations that will be more practical for analysis and patient management.[4]

CD38 is a marker associated with cellular activation and maturation in the hematopoietic cells.[16] Increased expression of CD38 is associated with advanced disease stage and poor response to chemotherapy.[15,17] Increase numbers of the peripheral lymphocytes, extensive bone marrow infiltration or atypical morphology of neoplastic cells can be observed in patients with a high CD38 expression. These patients also have a shorter overall survival probability.[16-18] In recent studies, leukemic cells with pre-germinal center phenotype were shown to have increased CD38 expression whereas the mutated cells demonstrated lower expression of the molecule. Therefore, it was suggested that IgVH mutation could be shown through the analysis of CD38 expression. On the other hand, IgVH mutation and increased CD38 expression are currently considered as independent prognostic factors.[19-21]

ZAP70 is a member of the tyrosine kinase family.[22,23] This molecule has been associated with B cell receptor signaling in CLL.[24] In leukemic cells, ZAP70 expression is associated with a worse prognosis when the expression levels exceed 20% of the baseline.[20] Studies demonstrated a negative correlation between IgVH mutation and ZAP70 expression, and ZAP70 expression was found to be increased in cases without IgVH mutation. It was suggested that ZAP70 was not only helpful in determining the mutation status, but could also be an important prognostic factor for overall survival and progression-free survival.[19] The analysis of ZAP70 is simpler and less expensive when compared to IgVH mutation analysis, and has been the preferred method in some studies.[25,26]

It is critical to determine IgVH mutation status of patients in order to determine prognosis and establish treatment in patients with CLL. In our study, we analyzed ZAP70 and CD38 expression in

bone marrow samples of CLL patients in order to establish the relationship of these markers with prognosis, and patterns of bone marrow involvement.

MATERIAL AND METHODS

SUBJECTS

Eighty-four cases of CLL diagnosed in Adnan Menderes University, Faculty of Medicine between 2007 and 2011 were retrospectively evaluated. Of 84 patients, 29 were females and 55 were males. The mean age of the patients was 65 years at the time of diagnosis (range: 35-89 years). The diagnosis of CLL was based on the criteria declared by Cancer Institute Working Group (NCI-WG).[27] After examining histopathological features in tissue preparations and cellular characteristics in Giemsa stained aspiration films and imprint preparations; CD3, CD5, CD10, CD20, CD 23, and cyclin-D1 expressions were evaluated, and the diagnosis was confirmed in each case. H&E stained preparations were evaluated again, and bone marrow involvement patterns (diffuse, nodular, interstitial and mixed) were determined.

IMMUNOHISTOCHEMICAL STAINING

ZAP70 (Santa Cruz Biotechnology, Sc-1526, 200 µg/ml, 1/400 dilution) and CD38 (Santa Cruz Biotechnology, Sc-7325, 200 µg/ml, 1/300 dilution) were investigated in new sections obtained from the paraffin-embedded blocks of the biopsy specimens. Immunohistochemical staining was made with Avidin-Biotin complex system. All examinations were made under a light microscope (Olympus BX51, Tokyo, Japan) at magnifications of x 10, 20 and 40. ZAP70 indicated cytoplasmic staining, and CD38 indicated membranous staining. Staining was scored by counting at least 200 tumor cells in neighboring tumor regions where the staining was the most intensive, and by calculating the ratio of stained ones to those not stained. The obtained values of <25% were scored as 0, 25-50% as 1, 51-75% as 2 and >75% as 3 for ZAP70 (Figure 1). For CD38, <10%, 11-20%, 21-30% and >30% were scored as 0, 1, 2 and 3, respectively (Figure 2). The cases with more than 25% of positive tumor cells were regarded as ZAP70 positive, and 30% of positive tumor cells were regarded as CD38 positive.[28]

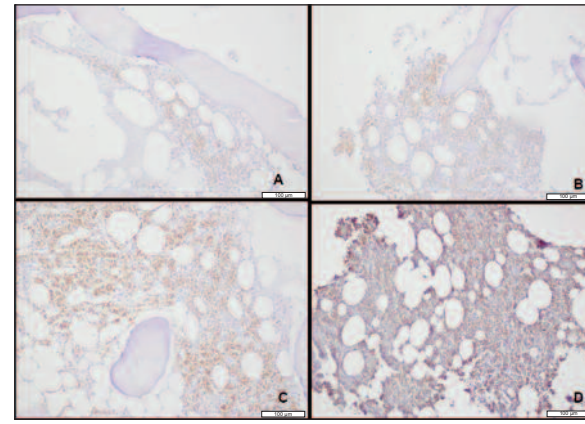

FIGURE 1: Staining for ZAP-70: A) <25% positivity, B) 25-50% positivity, C) 51-75% positivity, D) >75% positivity (Anti-ZAP-70, X200).
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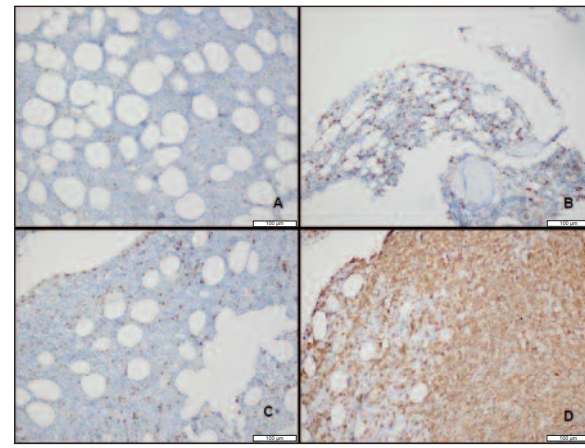

FIGURE 2: Staining for CD38: A) <10% positivity, B) 11-20% positivity, C) 21-30% positivity, D) >30% positivity (Anti-CD38, X200).
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

DNA ISOLATION AND IgVH MUTATION ANALYSIS

Five sections with 10 µm thickness were obtained from each paraffin-embedded tissue. DNA isolation in these sections was made with INVITROGEN QUANT-IT DSDNA FFPE Tissue Kit according to manufacturer's instructions. Following incubation of the sections with proteinase K at 56°C in the water bath, quantitative measurements of DNA were made with a spectrophotometer (ND-2000, Nanodrop, USA) in accordance with the kit protocol. Polymerase chain reaction (PCR) reaction and sequence analyses were made in 20 cases, and the

ideal value of DNA measurements was about 1.8 at spectrophotometer. BİOMED-2 primers were used to detect IgVH mutations (14q32.3) in all specimens.[29] Seven forward primers and one JH reverse primer of the FRII region of the V1-7 segments with an expected size from approximately 260bp were used to detect the mutations (Table 1). All primers were examined with conventional PCR. Seven PCR tubes containing one forward primer and one reverse primer were prepared for each patient. In total, 20 µl PCR mixture was obtained for each tube. PCR was performed as in the following order: The first denaturation was carried out at 94°C, for 7 minutes. It was followed by 35-cycle denaturation at 94°C for 1 minute, binding at 58°C for 1 minute and elongation at 72°C for 1.5 minutes. Following the last cycle, one final elongation at 72°C for 10 minutes was added. Thereafter, the specimens were kept at 4°C (Thermal Cycler BIO-RAD C1000). For imaging, 2%agarose gel (Prona Basic Agarose) was prepared. The electrophoresis tank was connected to a current of 100 volts and 2 amperes, for 30 minutes. Finally, multiplied bands were checked in the UV-transilluminator, and photographed (Vilber Lourmat jel imaging system) to compare with the markers. Band formation in the range of 250-300bp indicated the presence of mutation. No band formation was regarded as the absence of mutation (Figure 3).

Applied Biosystems 3130xl Genetic Analyzer was used for sequence analyses. Obtained sequences were compared with those in the databases, NCBI-Blast (http://www.ncbi.nlm.nih.gov/igblast/.) and VBASE (http://www.vbase.mrccpe.cam.ac.uk/). Sequences equal to and smaller than 98% were considered as homology mutations.

TABLE 1: Primers used in polymerase chain reaction analyses in this study.[29]

Forward primers			Sequence (5' to 3')
V_H1-FR2	(1-2)	(-192)	CTGGGTGCGACAGGCCCCTGGACAA
V_H2-FR2	(2-5)	(-190)	TGGATCCGTCAGCCCCCAGGGAAGG
V_H3-FR2	(3-7)	(-189)	GGTCCGCCAGGCTCCAGGGA
V_H4-FR2	(4-4)	(-188)	TGGATCCGCCAGCCCCCAGGGAAGG
V_H5-FR2)	(5-51)	(-190)	GGGTGCGCCAGATGCCCGGGAAAGG
V_H6-FR2	(6-1)	(-194)	TGGATCAGGCAGTCCCCATCGAGAG
V_H7-FR2	(7)	(-192)	TTGGGTGCGACAGGCCCCTGGACAA
Reverse primer			**Sequence (3' to 5')**
JH consensus		(+57)	CCAGTGGCAGAGGAGTCCATTC

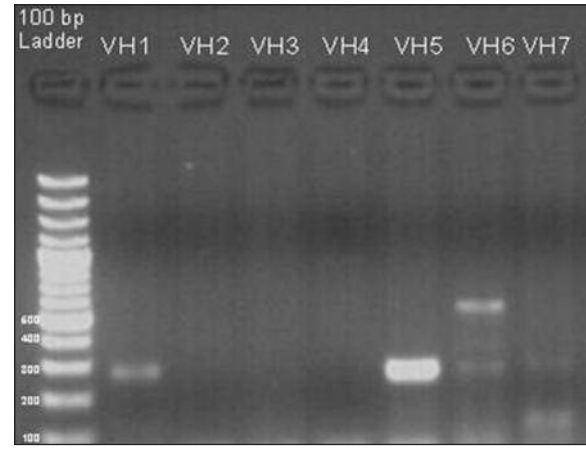

FIGURE 3: Image of gel in which band formation was observed at 250-300bp at VH1 and VH5 primers.

STATISTICAL ANALYSIS

Statistical analyses were made using Statistical Package for Social Sciences 14.0 for Windows. The descriptive statistics were given as mean, standard deviation, frequency and percentages. Chi-square test, Fisher-Freeman-Halton test and Fisher's exact test were used for categorical variables, and paired samples t-test was used for continuous variables. Pearson correlation test was used for determination of the correlations, and Kaplan Meier test was employed for survival statistics. $p<0.05$ was considered as significant.

RESULTS

Table 2 shows general characteristics of 84 CLL cases, retrospectively included in the study.

BONE MARROW INFILTRATION PATTERNS AND BINET CLINICAL STAGES

Involvement was diffuse in 38 patients (45.2%), interstitial in 29 patients (34.5%), nodular in 10 patients (11.9%) and mixed in 7 patients (8.3%). The patients with diffuse involvement of the bone marrow required significantly early treatment after the diagnosis (p=0.018), and had an advanced disease according to Binet clinical staging (p=0.036) (Figure 4).

TABLE 2: General characteristics of the patients.

	n=84 (%)
Mean Age (years)	65.36±12.5
Gender	29 Females (34.5%)
	55 Males (65.5%)
Binet Clinical stage	32 patients (38.1%) Stage A
	28 patients (33.3%) Stage B
	24 patients (28.6%) Stage C
Therapeutic needs after diagnosis	40 patients (47.6%)
Follow-up without treatment	44 patients (52.4%)
Prognosis	30 remission (35.7%)
	44 follow-up without treatment (52.4%)
	10 death (11.9%)
Bone marrow infiltration pattern	38 diffuse (45.2%)
	10 nodular (11.9%)
	29 interstitial (34.5%)
	7 mixed (8.3%)
ZAP70 (+/-) cut-off value 25%	45/39
CD38 (+/-) cut-off value 30%	25/59
IgVH mutation status (+/-) cut-off value 98%	6/20

According to Binet clinical staging, 32 patients (38.1%) had stage A disease, 28 patients (33.3%) had stage B disease, 24 patients (28.6%) had stage C disease. There was a significant correlation between clinical stage and the therapeutic needs, and the patients with advanced disease needed treatment significantly earlier (p<0.001) (Table 3).

ZAP70 AND CD38 EXPRESSIONS

Immunohistochemical staining with ZAP70 showed positive staining in 45 patients (53.6%) and negative staining in 39 patients (46.4%). The patients with ZAP70 staining were found to require treatment earlier after the diagnosis of their disease (p=0.046). Immunohistochemical staining with CD38 showed positive staining in 25 patients (29.8%) and negative staining in 59 patients (70.2%). There was a positive correlation between expression patterns of ZAP70 and CD38 (p<0.001

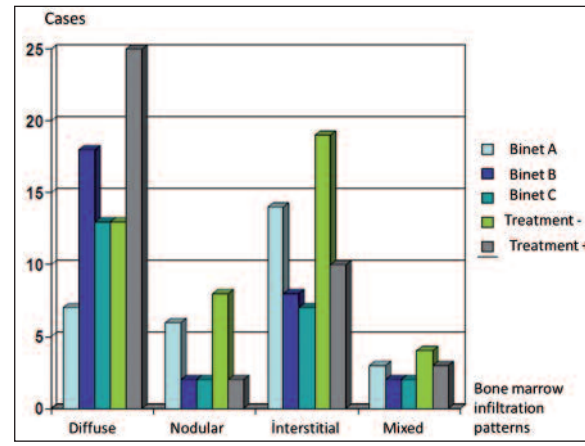

FIGURE 4: Distribution of Binet clinical stages and treatment statuses of patients in relation with bone marrow infiltration patterns.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

TABLE 3: Characteristics and statistical properties of patients according to the bone marrow infiltration pattern.

		Bone marrow infiltration pattern				
Characteristics	**Frequency (%)**	**Diffuse (%)**	**Nodular (%)**	**Interstitial (%)**	**Mixed (%)**	**P-value**
Gender						0.241
Female	29 (34.5)	15 (17.9)	5 (6.0)	6 (7.1)	3 (3.6)	
Male	55 (65.5)	23 (27.4)	5 (6.0)	23 (27.4)	4 (4.8)	
Binet Clinical stage						**0.036**
Stage A	32 (38.1)	7 (8.3)	6 (7.1)	16 (19.0)	3 (3.6)	
Stage B	28 (33.3)	18 (21.4)	2 (2.4)	6 (7.1)	2 (2.4)	
Stage C	24 (28.6)	13 (15.5)	2 (2.4)	7 (8.3)	2 (2.4)	
Treatment status						**0.018**
Therapeutic needs after diagnosis	40 (47.6)	25 (29.8)	2 (2.4)	10 (11.9)	3 (3.6)	
Follow-up without treatment	44 (52.4)	13 (15.5)	8 (9.5)	19 (22.6)	4 (4.8)	
ZAP70						**<0.001**
Positive patients	45 (53.6)	29 (34.5)	2 (2.4)	11 (13.1)	3 (3.6)	
Negative patients	39 (46.4)	9 (10.7)	8 (9.5)	18 (21.4)	4 (4.8)	
CD38						**<0.001**
Positive patients	25 (29.8)	20 (23.8)	2 (2.4)	2 (2.4)	1 (1.2)	
Negative patients	59 (70.2)	18 (21.4)	8 (9.5)	27 (32.1)	6 (7.1)	

r=0.606). The degree of staining with ZAP70 and CD38 was significantly higher in diffuse bone infiltrations compared to nodular, interstitial and mixed bone infiltrations (ZAP70 p<0.001; CD38 p<0.001) (Table 4).

IgVH MUTATION STATUS

Evaluation of IgVH mutation status in 20 patients showed VH5 rearrangement in one patient, VH3 rearrangement in four patients and VH6 rearrangement in one patient. VH5 (VH5-51) was used in one patient, and showed a homology of 96%. VH3 (VH3-21) was used in four patients, and showed homologies of 88%, 86%, 90% and 91%. VH6 (VH6-1) was used in one patient and showed a homology of 92%. Table 5 shows mutation status, bone marrow infiltration and immunohistochemical properties of six cases. One patient with mutation had died in the follow-up period. There was no significant difference between IgVH mutation status and Binet clinical stage (p=0.413), therapeutic needs after diagnosis of the disease (p=0.910), bone marrow infiltration (p=0.120) and ZAP70 (p=1.000) and CD38 (p=0.831) expressions.

SURVIVAL ANALYSIS

The mean survival was 13.452±12.714 months during 1-50 months of follow-up. Out of 84 patients, 40 (47.6%) required treatment after the diagnosis of their disease. Ten patients, of whom six were

TABLE 4: Characteristics and statistical properties of the patients in relation with ZAP70 expression.

		ZAP70 expression		
Characteristics	**Frequency (%)**	**ZAP70 positive patients (%)**	**ZAP70 negative patients (%)**	**P-value**
Gender				0.243
Female	29 (34.5)	13 (15.5)	16 (19.0)	
Male	55 (65.5)	32 (38.1)	23 (27.4)	
Binet Clinical stage				0.068
Stage A	32 (38.1)	12 (14.3)	20 (23.8)	
Stage B	28 (33.3)	18 (21.4)	10 (11.9)	
Stage C	24 (28.6)	15 (17.9)	9 (10.7)	
Treatment status				**0.046**
Therapeutic needs after diagnosis	40 (47.6)	26 (31.0)	14 (16.7)	
Follow-up without treatment	44 (52.4)	19 (22.6)	25 (29.8)	
Bone marrow infiltration pattern				**0.001**
Diffuse	38 (45.2)	29 (34.5)	9 (10.7)	
Nodular	10 (11.9)	2 (2.4)	8 (9.5)	
Interstitial	29 (34.5)	11 (13.1)	18 (21.4)	
Mixed	7 (8.3)	3 (3.6)	4 (4.8)	
CD38				**<0.001**
Positive patients	25 (29.8)	20 (23.8)	0(0.0)	
Negative patients	59 (70.2)	25 (29.8)	39 (46.4)	

TABLE 5: Mutation statuses, bone marrow infiltration patterns, and immunohistochemical properties of six patients.

Patient	ZAP70	CD38	Infiltration type	VH family	Germline	Homology %
1	-	-	Diffuse	VH3	VH3-21	88%
2	-	-	Mixed	VH5	VH5-51	96%
3	-	-	Diffuse	VH3	VH3-21	86%
4	+	+	Diffuse	VH6	VH6-1	92%
5	+	+	Interstitial	VH3	VH3-21	90%
6	+	-	Interstitial	VH3	VH3-21	91%

males and four were females, died within 1-46 months of the diagnosis (Figure 5). They were aged between 45 and 71 years. Out of ten patients dying despite receiving chemotherapy, six had interstitial, one had nodular, and three had diffuse bone marrow infiltrations. In addition, seven had stage C disease, and three had stage B disease according to Binet staging criteria. Seven had positive staining for ZAP70 expression, and four had positive staining for CD38. There was no significant relations between degrees of staining with ZAP70 (p=0.327) and CD38 (p=0.475), bone marrow infiltration patterns (p=0.366) and IgVH mutation status (p=0.520). However, there was a significant correlation between Binet clinical stage and death (p=0.017). In fact, all patients who died had advanced disease (Table 6).

DISCUSSION

CLL is the most common type of leukemia and a lymphoid malignancy, the outlook of which varies widely.[9,20] This tumor appears two times as frequently in men as in women, and the mean age of the patients is 65 years.[30] The mean age of 84 patients included in our study was 65.36±12.5 (± Standard deviation) years, and 65% and 53% of the patients were males and females, respectively, consistent with the literature. Recently, there has been an increase in the number of young people diag-

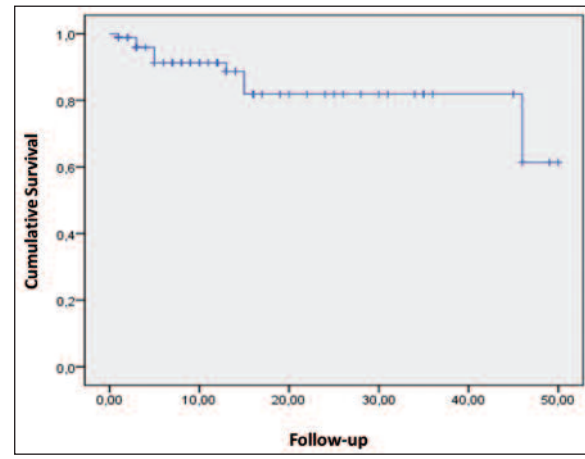

FIGURE 5: Cumulative survival curves.

TABLE 6: Characteristics and statistical properties of the patients in relation with their prognosis.

Characteristics	Frequency (%)	Prognosis		p
		Alive (%)	Dead (%)	
Gender				0.170
Female	29 (34.5)	27 (32.1)	2 (2.4)	
Male	55 (65.5)	347 (56.0)	8 (9.5)	
Binet Clinical stage				**0.017**
Stage A	32 (38.1)	30 (35.7)	2 (2.4)	
Stage B	28 (33.3)	27 (32.1)	1 (1.2)	
Stage C	24 (28.6)	17 (20.2)	7 (8.3)	
Treatment status				**0.042**
Therapeutic needs after diagnosis	40 (47.6)	32 (38.1)	8 (9.5)	
Follow-up without treatment	44 (52.4)	42 (50.0)	2 (2.4)	
Bone marrow infiltration pattern				0.366
Diffuse	38 (45.2)	35 (41.7)	3 (3.6)	
Nodular	10 (11.9)	9 (10.7)	1 (1.2)	
Interstitial	29 (34.5)	23 (27.4)	6 (7.1)	
Mixed	7 (8.3)	7(8.3)	0(0.0)	
CD38				0.475
Positive patients	25 (29.8)	21 (25.0)	4 (4.8)	
Negative patients	59 (70.2)	53 (63.1)	6 (7.1)	
ZAP70				0.327
Positive patients	45 (53.6)	38 (45.2)	7 (8.3)	
Negative patients	39 (46.4)	36 (42.9)	3 (3.6)	

nosed with CLL.[4,13,15] In our study, 25 patients were under the age of 55 years when they were diagnosed with CLL.

Prognosis varies among patients, and the mean survival is ten years.[31] There are two clinical staging systems for CLL, described by Rai and Binet.[32,33] There is a significant correlation between the disease stage and survival according both systems. However, these systems are not useful for differentiation of progressive and slow clinical courses of the disease, when the stage of the disease is not taken into account. In our study, Binet clinical stage was significantly correlated with overall survival, and all patients who died had an advanced disease stage. In addition, the clinical stage was significantly correlated with therapeutic needs after the diagnosis of the disease, and all patients with advanced disease required treatment earlier. a number of studies for performed to determine the prognostic factors in CLL.[34] Lymphocyte doubling time, serum ß2 microglobulin levels, soluble CD23, serum timidin kinase, bone marrow infiltration patterns, and cytogenetic anomalies were studied. However, they were not sensitive enough to differentiate stable from progressive forms of the disease in the early stages.[35]

Although bone marrow biopsy is usually not essential for the diagnosis of CLL, it is very useful for evaluation of the clinical course and response to treatment. It also helps to confirm the diagnosis of CLL with an atypical morphology, and offers information about infiltration types.[31] It has been reported that lymphoid infiltration patterns in bone marrow biopsy specimens can be considered as a prognostic factor in CLL. Several studies suggested that diffuse infiltration was associated with a poor prognosis, and interstitial and nodular infiltrations were associated with a favorable prognosis.[4,24,36] In our study, ZAP70 and CD38 expressions were significantly higher in the patients with diffuse infiltrations. Besides, these patients needed treatment earlier after the diagnosis of their disease, and had advanced stages of the disease according to Binet clinical staging. When only bone marrow infiltration type was used as a prognostic marker, some CLL cases with a favorable clinical course can be accidentally considered as the ones at a serious risk.[35]

There has been an increasing interest in the investigation of biological markers that could indicate the prognosis of CLL in its early stages. As a result, many biological markers of prognostic importance have been determined recently. IgVH mutation status worths considering in a different way since it sheds light on pathogenesis of CLL, and helps to determine prognosis.[37] IgVH mutation is the most reliable prognostic marker to be used to identify two subgroups of CLL, namely, M-CLL and U-CLL.[24] In general, M-CLL has a slow course, and the mean survival of the patients with a mild treatment or without treatment is 25 years. However, U-CLL has a rapid course, and the mean survival of the patients is 8 years.[13,15] In cases of M-CLL, IgVH4-34, IgVH3-7 and IgVH3-23 are frequently used, but in cases of U-CLL, IgVH1-69 is frequently used.[37] M-CLL cases harboring VH3-21 gene are considered beyond the general classification of CLL, and have short survival independent of mutation status.[38] Recent studies have shown that patients with progressive CLL do not have mutations, and have considerably short survivals and short duration of treatment after diagnosis when the cut-off values were considered as 98%.[39-41] In our study, in the cases with mutations, VH5-51, VH3-21 and VH6-1 genes were used, and the most frequently used was VH3-21 gene. Unlike the finding that M-CLL had a slow clinical course and a favorable prognosis in other studies, we found that the cases displaying mutation had in Binet stage B and C and required treatment early after diagnosis, which can be attributed to the fact that the rate of VH3-21 gene mutation was higher, associated with short survival. However, larger studies are needed to prove that this new prognostic marker is useful to determine the course and the progression of the disease.

Due to presence of significant differences between M-CLL and U-CLL, large studies focused on the use of VH/VL gene and mutation status. These studies showed a higher tendency to use some members of IgVH gene family and higher mutation rates among certain IgVH genes.[42] These findings suggested clonal enlargement and differentiation of leukemic B cells in reaction to unidentified foreign antigens or to their own antigens. In fact, environmental and ethnic variables may affect the use of

IgVH gene in CLL.[40] Most of the studies conducted so far have been on Western societies with similar geographic and ethnic characteristics, and VH3-21 was the most frequently used gene in Scandinavian countries (11.7%), Ireland (7.9%) and Mediterranean countries (2-3%). There is little information about CLL in Asian countries. There are only a few small studies in Japan, China and Iran.[39,40,43] This can be due to the lower incidence of CLL in Asian populations compared to Western countries. The skewness for the use of IgVH gene in CLL patients from different ethnic populations suggests that pathogens and autoantigens which have not been identified yet may play a role in the development of CLL.[40]

IgVH mutation analysis is a sensitive and independent prognostic marker used to classify patients in clinical studies. Although guidelines have been prepared for IgVH mutation analysis, the procedure requires intensive laboratory work, which is the most important barrier to its use in practice.[37] Besides, it is quite difficult and expensive to use in routine laboratory investigations. This is why there is a need to find new markers that can show mutations.[9,16] Now, ZAP70 and CD38 seem to be leading candidates for it.[37]

Many studies investigated the pathobiological role and expression of CD38 in CLL. A higher rate of CD38 expression was associated with a poor clinical prognosis.[44] Damle et al. found that CLL patients with high CD38 expression did not have IgVH mutation, but IgVH mutation was correlated with favorable prognosis in patients with a low CD38 expression, and suggested that CD38 could be a marker likely to replace IgVH mutation status in CLL, which is supported by other studies.[15,35] Morabito et al. reported that CD38 expression over 30% was associated with U-CLL.[45] It was also proposed that presence of CD38 expression was associated with other markers of poor prognosis in CLL, namely, ZAP70, cytogenetic anomalies, serum CD23, serum β2 microglobulin, p53 function and cell size.[46] Domingo-Domenech et al. found that patients displaying CD38 positivity needed treatment more frequently compared to those without CD38 expression.[47] In our study, most of the patients having CD38 expression required treatment early after the diagnosis of their disease, but there was no statistically significant correlation.

In the recent years, there has been an interest in the idea that ZAP70 can be used instead of IgVH mutation, which will help to identify patients with an aggressive course of the disease.[12] Normally not expressed by B lymphocytes, this marker has been detected in CLL cells, and is useful not only in detection of mutation, but also in prediction of the prognosis. Its expression has been reported to be constant during the disease.[4,41] In studies completed so far, there has been a consistency of over 90% between ZAP70 and mutation analysis determining prognosis.[37] Rassenti et al. found that ZAP70 was the most sensitive risk factor for determining the time relapsing to the first treatment.[20] Shorter overall survival was reported in patients with ZAP70 positivity compared to those with ZAP70 negativity.[14,20,48] In a study by Weinberg et al., survival was shown to be shorter in patients with ZAP70 positivity, though not significant.[49] In our study, most of the dead patients had ZAP70 expression, but it was not statistically significant.

It was suggested in the literature that patients with ZAP70 expression needed treatment at the time of diagnosis. Weinberg et al. showed no correlation between ZAP70 and treatment-free follow-up.[49] In their study on 307 CLL patients, Rassenti et al. found that the mean treatment-free survival was 9.2 years in patients without ZAP70 expression, and 2.9 years in patients with ZAP70 expression.[20] ZAP70 expression turned out to be an important predictor of the need for treatment.[20] Düriq et al. also reported that patients with ZAP70 expression required more intensive chemotherapy than those without ZAP70 expression.[14] In our study, there was a significant difference between ZAP70 expression and need for treatment after diagnosis, and patients displaying ZAP70 staining required treatment earlier after the diagnosis of the disease.

Overexpression of ZAP70 is an independent negative prognostic factor, and it is correlated with CD38 expression and lack of IgVH mutation.[45] Determining CD38 and ZAP70 expressions is quicker and cheaper compared to IgVH mutation. Some reports showed that analysis of ZAP70 and CD38 si-

multaneously gave more valuable information than analysis of each one.[14,50] Schroers et al. showed that the clinical prognosis of the disease was poor in the presence of ZAP70 and CD38 expressions.[51] In recent years, Khoudoleeva et al. evaluated ZAP70 and CD38 expressions together in blood, bone marrow, lymph node and spleen specimens of CLL patients, and showed a positive correlation between ZAP70 and CD38 expressions in blood and bone marrow specimens.[44] Positive correlation was found between ZAP70 and CD38 expressions and clinical stage of the disease by Rivkina et al.[52] They emphasized that evaluation of ZAP70 and CD38 expressions together provided information about the possible stage of the disease.[52] In our study, these two markers were used together, and there was a positive correlation between ZAP70 and CD38 expressions.

Although clinical staging systems have limitations in terms of identification of patients with a disease likely to show progression in its early stages, they are conventionally used in prediction of disease prognosis. The findings of this study support a significant relation between clinical stage and survival. Although bone marrow biopsy is not necessary to diagnose CLL, it is quite useful in evaluation of clinical prognosis and response to treatment. The results of this study showed that diffuse infiltration in the bone marrow was a risk factor for disease progression. It can be used as an additional prognostic factor in CLL patients. It is easy and inexpensive to determine ZAP70 and CD38 expressions in CLL. However, this technique has some limitations. In fact, it is not quantitative and its evaluation is subjective. In contrast, it is more inexpensive and quicker to determine CD38 and ZAP70 expressions than IgVH mutation. Analysis of ZAP70 and CD38 in combination offers a more refined prognostic information. The findings of this study support the idea that ZAP70 and CD38 can be used as prognostic markers in CLL. ZAP70 is invaluable since it helps to determine patients who would need treatment in the future. Therefore, patients with ZAP70 expression should be followed more closely since their disease may show a rapid progression, and since these patients require earlier treatment. Using IgVH mutation analysis in routine is not appropriate yet. Further studies with larger sample sizes are needed to show whether this marker can be useful in prediction of disease prognosis and progression. In addition, findings from retrospective studies should be corroborated with prospective and controlled clinical studies that aim achieving more reliable prognostic scores.

REFERENCES

1. Ghia P, Ferreri AM, Caligaris-Cappio F. Chronic lymphocytic leukemia. Crit Rev Oncol Hematol 2007;64(3):234-46.
2. Kay NE, O'Brien SM, Pettitt AR, Stilgenbauer S. The role of prognostic factors in assessing 'high-risk' subgroups of patients with chronic lymphocytic leukemia. Leukemia 2007;21(9):1885-91.
3. Durak B, Akay OM, Aslan V, Ozdemir M, Sahin F, Artan S, et al. Prognostic impact of chromosome alterations detected by FISH in Turkish patients with B-cell chronic lymphocytic leukemia. Cancer Genet Cytogenet 2009;188(2):65-9.
4. Schade U, Bock O, Vornhusen S, Jäger A, Büsche G, Lehmann U, et al. Bone marrow infiltration pattern in B-cell chronic lymphocytic leukemia is related to immunoglobulin heavy-chain variable region mutation status and expression of 70-kd zeta-associated protein (ZAP-70). Hum Pathol 2006;37(9):1153-61.
5. Dighiero G, Hamblin TJ. Chronic lymphocytic leukaemia. Lancet 2008;371(9617):1017-29.
6. Moreno C, Montserrat E. New prognostic markers in chronic lymphocytic leukemia. Blood Rev 2008;22(4):211-9.
7. Gribben JG. Molecular profiling in CLL. In: Gewirtz AM, Muchmore EA, Burns LJ, eds. American Society of Hematology Education Book. 1st ed. Washington: American Society of Hematology; 2008. p.444-9.
8. Byrd JC, Stilgenbauer S, Flinn IW. Chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2004:163-83. doi:10.1182/asheducation-2004.1.163
9. Shanafelt TD, Geyer SM, Kay NE. Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL. Blood 2004;103(4):1202-10.
10. Zenz T, Mertens D, Döhner H, Stilgenbauer S. Molecular diagnostics in chronic lymphocytic leukemia - pathogenetic and clinical implications. Leuk Lymphoma 2008;49(5):864-73.
11. Van Bockstaele F, Verhasselt B, Philippé J. Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. Blood Rev 2009;23(1):25-47.
12. Orchard JA, Ibbotson RE, Davis Z, Wiestner A, Rosenwald A, Thomas PW, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004;363(9403):105-11.
13. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94(6):1848-54.
14. Dürig J, Nückel H, Cremer M, Führer A, Halfmeyer K, Fandrey J, et al. ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. Leukemia 2003;17(12):2426-34.
15. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94(6):1840-7.

16. Karhu R, Tobin G, Thunberg U, Vilpo L, Sundström C, Knuutila S, et al. More extensive genetic alterations in unmutated than in hypermutated cases of chronic lymphocytic leukemia. Genes Chromosomes Cancer 2003;37(4):417-20.

17. Deaglio S, Capobianco A, Bergui L, Dürig J, Morabito F, Dührsen U, et al. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. Blood 2003;102(6):2146-55.

18. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101(12):4944-51.

19. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348(18):1764-75.

20. Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med 2004;351(9):893-901.

21. Ibrahim S, Keating M, Do KA, O'Brien S, Huh YO, Jilani I, et al. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood 2001;98(1):181-6.

22. Chan AC, Irving BA, Fraser JD, Weiss A. The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc Natl Acad Sci U S A 1991;88(20):9166-70.

23. Latour S, Veillette A. Proximal protein tyrosine kinases in immunoreceptor signaling. Curr Opin Immunol 2001;13(3):299-306.

24. Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2002;100 (13):4609-14.

25. Korać P, Ajduković R, Kardum Paro MM, Jaksić B, Dominis M. Immunohistochemical analysis of ZAP-70 expression in chronic lymphocytic leukemia. J Mol Histol 2009;40(1):81-6.

26. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 2001;194(11):1639-47.

27. Cheson BD, Bennett JM, Grever M, Kay N, Keating MJ, O'Brien S, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 1996;87(12): 4990-7.

28. Dabbs D. Immunohistology of Non-Hodgkin Lymphoma. Diagnostic Immunohistochemistry Theranostic and Genomic Aplications. 3rd ed. Philadelphia: Saunders Elsevier; 2010. p.169-70.

29. van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17(12):2257-317.

30. Müller-Hermelink HK, Montserrat E, Catovsky D, Harris NL. Chronic lymphocyticleukemia/small lymphocytic lymphoma. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization Classification of Tumours. Pathology & Genetics Tumours of Haemapoietic and Lymphoid Tissues. Lyon: IARC Press; 2001. p.127-37.

31. Bojarska-Junak A, Roliński J, Kawiak J. Modification of immunocytochemical ZAP-70 assay for potential clinical application in B-cell chronic lymphocytic leukemia. Folia Histochem Cytobiol 2005;43(1):19-23.

32. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood 1975;46(2): 219-34.

33. Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 1981;48(1):198-206.

34. Chronic lymphocytic leukemia: recommendations for diagnosis, staging, and response criteria. International Workshop on Chronic Lymphocytic Leukemia Ann Intern Med 1989;110(3):236-8.

35. Del Poeta G, Maurillo L, Venditti A, Buccisano F, Epiceno AM, Capelli G, et al. Clinical significance of CD38 expression in chronic lymphocytic leukemia. Blood 2001;98(9):2633-9.

36. Hayat A, O'Brien D, O'Rourke P, McGuckin S, Fitzgerald T, Conneally E, et al. CD38 expression level and pattern of expression remains a reliable and robust marker of progressive disease in chronic lymphocytic leukemia. Leuk Lymphoma 2006;47(11):2371-9.

37. Yağcı M. [The significance of immunglobulin heavy chain gene mutations in chronic lymphocytic leukemia]. Turkiye Klinikleri J Hem Onc-Special Topics 2009;2(2):35-40.

38. Tobin G, Thunberg U, Johnson A, Thörn I, Söderberg O, Hultdin M, et al. Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood 2002;99(6): 2262-4.

39. Chen L, Zhang Y, Zheng W, Wu Y, Qiao C, Fan L, et al. Distinctive IgVH gene segments usage and mutation status in Chinese patients with chronic lymphocytic leukemia. Leuk Res 2008;32(10):1491-8.

40. Hojjat-Farsangi M, Jeddi-Tehrani M, Razavi SM, Sharifian RA, Mellstedt H, Shokri F, et al. Immunoglobulin heavy chain variable region gene usage and mutational status of the leukemic B cells in Iranian patients with chronic lymphocytic leukemia. Cancer Sci 2009;100(12):2346-53.

41. Admirand JH, Knoblock RJ, Coombes KR, Tam C, Schlette EJ, Wierda WG, et al. Immunohistochemical detection of ZAP70 in chronic lymphocytic leukemia predicts immunoglobulin heavy chain gene mutation status and time to progression. Mod Pathol 2010;23(11):1518-23.

42. Messmer BT, Raphael BJ, Aerni SJ, Widhopf GF, Rassenti LZ, Gribben JG, et al. Computational identification of CDR3 sequence archetypes among immunoglobulin sequences in chronic lymphocytic leukemia. Leuk Res 2009;33(3):368-76.

43. Nakahashi H, Tsukamoto N, Hashimoto Y, Koiso H, Yokohama A, Saitoh T, et al. Characterization of immunoglobulin heavy and light chain gene expression in chronic lymphocytic leukemia and related disorders. Cancer Sci 2009;100(4): 671-7.

44. Khoudoleeva O, Gretsov E, Barteneva N. Proliferative index and expression of CD38, Zap-70,and CD25 in different lymphoid compartments of chronic lymphocytic leukemia patients. Pathol Lab Med Int 2011;3:7-16.

45. Morabito F, Mangiola M, Oliva B, Stelitano C, Callea V, Deaglio S, et al. Peripheral blood CD38 expression predicts survival in B-cell chronic lymphocytic leukemia. Leuk Res 2001;25(11):927-32.

46. Deaglio S, Aydin S, Vaisitti T, Bergui L, Malavasi F. CD38 at the junction between prognostic marker and therapeutic target. Trends Mol Med 2008;14(5):210-8.

47. Domingo-Domènech E, Domingo-Clarós A, Gonzàlez-Barca E, Beneitez D, Alonso E, Romagosa V, et al. CD38 expression in B-chronic lymphocytic leukemia: association with clinical presentation and outcome in 155 patients. Haematologica 2002;87(10):1021-7.

48. Kröber A, Seiler T, Benner A, Bullinger L, Brückle E, Lichter P, et al. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood 2002;100(4):1410-6.

49. Weinberg JB, Volkheimer AD, Chen Y, Beasley BE, Jiang N, Lanasa MC, et al. Clinical and molecular predictors of disease severity and survival in chronic lymphocytic leukemia. Am J Hematol 2007;82(12):1063-70.

50. Hus I, Podhorecka M, Bojarska-Junak A, Roliński J, Schmitt M, Sieklucka M, et al. The clinical significance of ZAP-70 and CD38 expression in B-cell chronic lymphocytic leukaemia. Ann Oncol 2006;17(4):683-90.

51. Schroers R, Griesinger F, Trümper L, Haase D, Kulle B, Klein-Hitpass L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005;19(5):750-8.

52. Rivkina A, Vitols G, Murovska M, Lejniece S. Identifying the stage of new CLL patients using TK, ZAP-70, CD38 levels. Exp Oncol 2011; 33(2):99-103.