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Prognostic Significance of Apoptosis Regulators in B-Cell Chronic Lymphocytic Leukemia

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Abstract

Background: High levels of MCL-1 and BCL-2 proteins have been found in Chronic Lymphocytic Leukemia (CLL), and inversely correlated with response to treatment. BCL-2/Bax ratio is the main director of apoptosis in CLL. The study aimed to clarify the prognostic role of MCL-1, BCL-2 and BCL-2/Bax ratio in B-CLL. **Patients & method**: Estimation of MCL-1, BCL-2 and Bax expressions by a flow cytometry in 45 B-CLL patients and the prognostic value of these markers were correlated with other well-known established prognostic markers and treatment response. **Results**: MCL-1 was expressed in 60% of cases while BCL-2 was expressed in 82.2% of cases. MCL-1 expression was significantly high in male gender, short lymphocyte doubling time (LDT), and high expression of CD 38 ($p < 0.001$). High (Serum LDH, serum β2M, CD38 expression), low ZAP-70 expression, splenomegaly and higher Rai stage were significantly increased in patients with high expression of BCL-2 ($p < 0.001$); also a significant decrease in (HB level, platelet count), and increase in serum LDH, serum β2M, high C-D38 expression, low ZAP-70 expression, the poor cytogenetic and splenomegaly in patients with high expression of BCL-2/Bax ratio ($p < 0.001$). Among the 39 patients who started treatment when indicated responding patients had statistically significant lower BCL-2/Bax ratio than non-responding patients, although their lower mean of MCL-1 and BCL2 expressions values were insignificant. **In conclusion:** MCL-1, BCL-2 expressions and BCL-2/Bax ratio could be useful potential predictive and prognostic markers in B-CLL.

Keywords

Myeloid Cell Leukemia 1, B-Cell Lymphoma 2, BAX, B-Cell Chronic Lymphocytic Leukemia

1. Introduction

The most prevalent form of adult leukemia is B-Chronic lymphocytic leukemia (B-CLL); it is characterized by accumulation of CD5+ and CD23+ B-cell lymphocytes [1]. It is a heterogeneous disease with variable clinical course; some patients presented with an indolent disease and need no or little treatment, while others have aggressive diseases at diagnosis. Rai and Binet staging systems cannot predict the disease outcome in early stages of Chronic Lymphocytic Leukemia [2]. Additionally to known prognostic markers, other markers like V region genes mutation, genomic aberrations, and expression of CD38 are counted as significant prognostic markers [3]. Although response can be attained in 60% of patients by existing treatment, the disease is still not curable [4]. This incurability is mainly caused by CD5+ B cells accumulation which is responsible for cell immortality [5]. Recently, it is recommended that survival signals' modulation interferes with apoptosis which possibly plays the vital tool in CLL pathogenesis. BCL-2 (B-cell lymphoma-2) family of anti-apoptotic (BCL-xl, BCL-2, MCL-1 and BCL-w) and proapoptotic (bok, bax, and bak) proteins are essential for apoptosis control in CLL [6]. BCL-2 claimed as the major protein in CLL which helps for survival prediction [7]. Some studies showed that high BCL-2 levels and low expression of bax protein are connected to treatment resistant in CLL cells [8]. Numeroustrials established that in CLL; the equilibrium between members of pro- and anti-apoptotic BCL-2 family impacts the sensitivity to chemotherapy and patients survival [9]; also it is found that this balance between anti- and pro-apoptotic proteins is more important than intensity of each protein expression [10]. BCL-2/Bax ratio was identified as the main determinant in regulation of apoptosis in CLL by some investigators [11] [12], while others denied this [13]. In our study, we evaluated the effect of MCL-1, BCL-2, and BCL-2/Bax ratio on tumor response, survival, and relation to other established markers in CLL.

2. Method

2.1. Subjects and Methods

We enrolled 45 denovo B-CLL patients, who were presented to Clinical pathology, Medical Oncology, Clinical oncology and Internal Medicine departments, Zagazig University Hospitals. In the period between June 2013 and May 2015, complete history taking was done for all patients, clinical examination, and laboratory investigations; CBC, direct Coombs' test, LDH and β2M estimation.

An international scoring system based on expression patterns of immunophenotyping markers in CLL has been established. Patients with Chronic B lymphoproliferative disorders were diagnosed by evidence of persistent absolute lymphocytosis > 5000/μl (5×10^9/liter) for 3 months. Immunophenotyping was performed by monoclonal antibodies panels: CD3, CD7, CD5, CD19, CD20, CD22, K/λ, CD23, CD79b, and FMC7, also estimation of cytoplasmic ZAP-70 expression and surface CD38 [14]. Anti-ZAP-70-FITC (Becton-Dickinson, USA and Bi-

oscience) with an intrastain kit (Dako) and anti-CD38-PE (DAKO, CA, USA) were provided with a permeabilizing agent. For FITC, PC-5 and PE (negative control) conjugated monoclonal antibody specific isotypic controls were used. We selected lymphocytes in the forward scatter against side scatter dot blot and gated as CD19/CD5 positive cells. Samples analysis was performed by multicolor FCM (FACS Caliber flow cytometry Becton Dickinson, USA). The percentage of gated cells was measured as positive expression over the corresponding isotypic control with cut-off ≥30% for CD38 and ≥20% for ZAP-70.

2.2. Special Investigations

Flow cytometric analysis of MCL-1, BCL-2 and Bax expressions by using FITC-conjugated MoAb (Dako, Glostrup, Denmark). Peripheral blood samples (EDTA blood) were tested initially with diagnosis or before any treatment. All samples were initially incubated with CD19 PE and CD5 APC MoAbs for 30 min at 4˚C. Subsequently the cells after washing twice in PBS were fixed and permeabilized with Cell Permeabilizationkit. Samples were then incubated at 4˚C for 30 min with 10 uL anti-MCL-1, anti-BCL-2, and anti BAX FITC conjugated. We evaluated MCL-1, BCL-2 and Baxas relative mean fluorescence intensities (rMFIs). The ratio between BCL-2 and Bax (rMFIs), and non-specific MoAb MFI on B cells also were calculated. In current study, a cut-off of 25% of positive cells was chosen to determine MCL-1 positive in CLL cases [15] and a cut-off of 10% of positive cells was chosen to discriminate BCL-2 and BAX positive from negative in CLL cases [16].

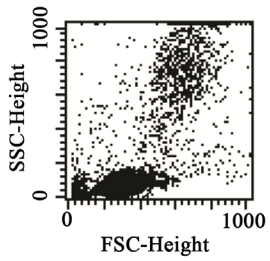
Figure 1(a) shows a flowcytometric histogram for positive markers CD23, CD19, CD22 with co-expression between CD5and CD20 with K/L showed dim expression, and also shows that; MCL-1, BCL-2 and CD38 are positive. **Figure 1(b)** shows a flow cytometric histogram for positive markers CD23, CD19, CD22 with co-expression between CD5and CD20; this is a case of B-CLL. And also shows that MCL-1, BCL-2 and CD38 are negative. Separation of two subgroups with different OS and/or PFS was done depending on BCL-2/Bax-2 ratio, probabilities was calculated by different methods like Youden's index, receiver-operating characteristic (ROC) analysis and median values. We concluded BCL-2/Bax median value ≥ 1.50 (range 0.27 - 6.10) as the threshold value and this was confirmed by ROC analysis Online Supplementary, **Figure 2**.

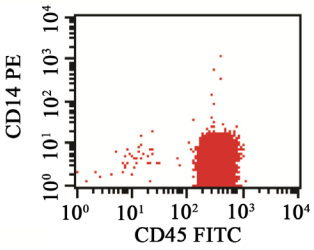
2.3. Cytogenetic Analysis

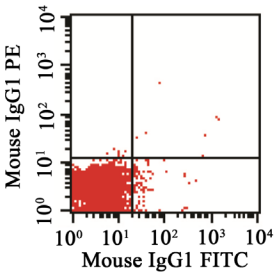
By using fluorescence in situ hybridization (FISH) technique, Cytogenetic analysis was done on peripheral blood, a locus specific identifier DNA probe (LSI) Kit was used, Vysis (Abbott Park, Ill, USA).

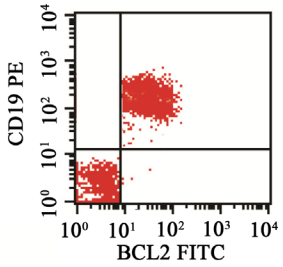
2.4. When to Initiate Therapy

There are indications to begin treatment for CLL patients including: elevated total leucocytes count with a lymphocytes doubling time < 12 months, having anemia or thrombocytopenia as a result of bone marrow infiltration, development



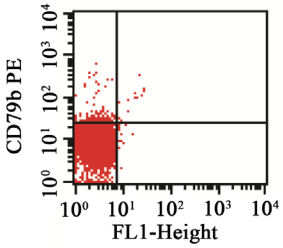






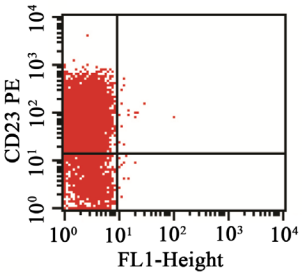
Tubu: BCL2/CD19
Quad Location: 8, 12

Quad	% Gated	% Total
UL	0.30	0.22
UR	90.11	67.17
LL	9.46	7.05
LR	0.13	0.10



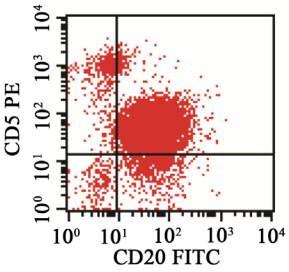
Tubu: CD79B
Quad Location: 8, 21

Quad	% Gated	% Total
UL	6.03	4.75
UR	0.18	0.14
LL	93.62	73.72
LR	0.17	0.13



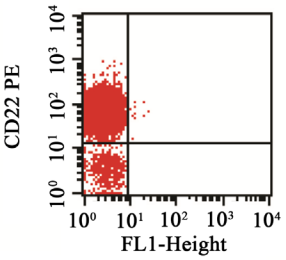
Tubu: CD23
Quad Location: 9, 12

Quad	% Gated	% Total
UL	84.34	67.22
UR	0.28	0.22
LL	15.23	12.14
LR	0.15	0.12



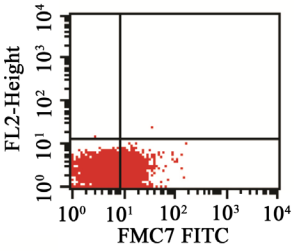
Tubu: CD20/CD5
Quad Location: 9, 12

Quad	% Gated	% Total
UL	5.46	4.34
UR	84.02	66.83
LL	2.20	1.75
LR	8.32	6.62



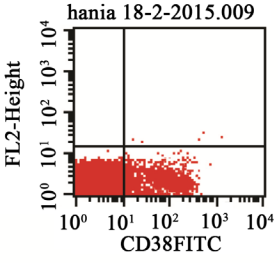
Tubu: CD22
Quad Location: 9, 12

Quad	% Gated	% Total
UL	92.55	75.83
UR	0.15	0.12
LL	7.30	5.98
LR	0.00	0.00



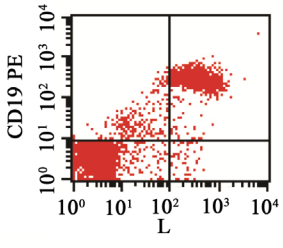
Tubu: FMC7
Quad Location: 9, 12

Quad	% Gated	% Total
UL	0.01	0.01
UR	0.02	0.02
LL	67.91	54.37
LR	32.05	25.66



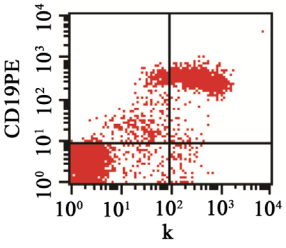
Tubu: CD38
Quad Location: 11, 13

Quad	% Gated	% Total
UL	0.00	0.00
UR	0.08	0.06
LL	91.43	72.99
LR	38.49	36.78



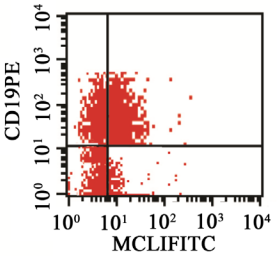
Tubu: L/CD19PE
Quad Location: 94, 8

Quad	% Gated	% Total
UL	8.89	3.25
UR	10.00	5.94
LL	51.49	18.82
LR	1.48	0.54



Tubu: K/L/CD19PE
Quad Location: 94, 8

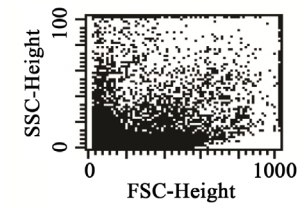
Quad	% Gated	% Total
UL	11.93	4.36
UR	35.10	12.83
LL	51.22	18.72
LR	1.75	0.64

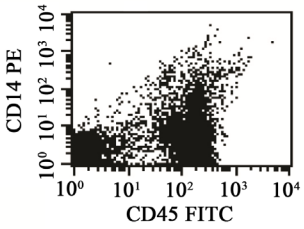


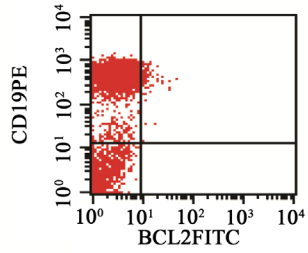
Tubu: MCL1 FITC/CD19PE
Quad Location: 6, 11

Quad	% Gated	% Total
UL	20.08	8.01
UR	70.65	50.08
LL	9.74	1.19
LR	10.53	2.94

(a)

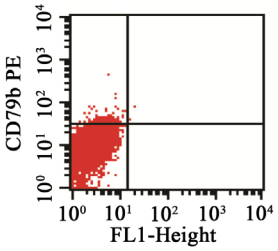






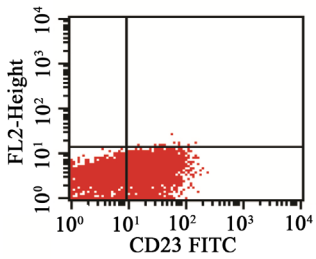
Tubu: BCL2/CD19
Quad Location: 9, 12

Quad	% Gated	% Total
UL	79.02	40.49
UR	2.73	1.40
LL	18.23	9.34
LR	0.02	0.01



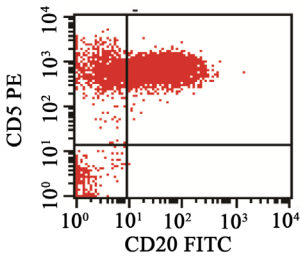
Tubu: CD79B
Quad Location: 14, 27

Quad	% Gated	% Total
UL	4.21	2.38
UR	0.07	0.04
LL	95.71	54.15
LR	0.02	0.01



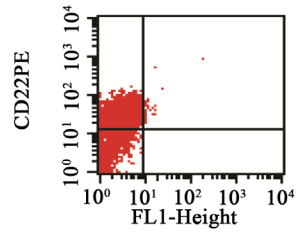
Tubu: CD23
Quad Location: 9, 12

Quad	% Gated	% Total
UL	0.05	0.03
UR	0.88	0.50
LL	33.09	18.76
LR	65.97	37.40



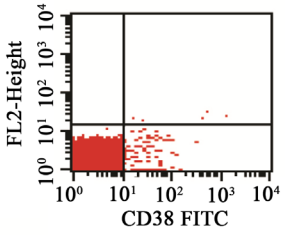
Tubu: CD20/CD5
Quad Location: 9, 12

Quad	% Gated	% Total
UL	19.45	11.01
UR	75.79	42.91
LL	4.75	2.69
LR	0.02	0.01



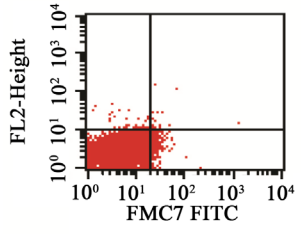
Tubu: CD22
Quad Location: 9, 12

Quad	% Gated	% Total
UL	76.05	39.73
UR	0.94	0.49
LL	23.01	12.02
LR	0.00	0.00



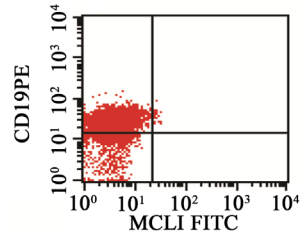
Tubu: CD38
Quad Location: 11, 13

Quad	% Gated	% Total
UL	0.00	0.00
UR	0.08	0.06
LL	91.43	72.99
LR	8.49	6.78



Tubu: FMC7
Quad Location: 19, 9

Quad	% Gated	% Total
UL	1.97	1.10
UR	0.65	0.36
LL	93.40	52.11
LR	3.98	2.22



Tubu: MCL1 FITC/CD19PE
Quad Location: 21, 13

Quad	% Gated	% Total
UL	88.36	82.89
UR	0.26	0.24
LL	11.38	10.68
LR	0.00	0.00

(b)

Figure 1. (a) Flow cytometric dot plots of BCL-2, MCL1 and bax on CD19+ cells on peripheral blood sample of one negative representative CLL case with reported MFI (mean fluorescent intensity) values; (b) Flow cytometric dot plots of BCL-2, MCL1 and bax on CD19+ cells on peripheral blood sample of one positive representative CLL case with reported MFI values.

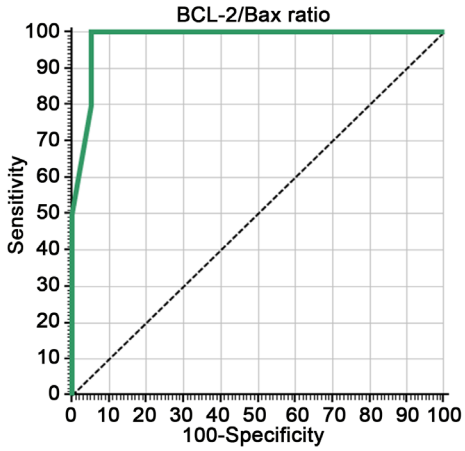


Figure 2. Receiver operating characteristic (ROC) curve of BCL-2/Bax ratio as a predictor of overall response of CLL patients to treatment.

of systemic B-symptoms, bulky lymphadenopathy, increasing organomegally, and recurrent infection. For patients <60 years fludarabine/cyclophos-phamide + Rituximab (FCR) protocol was given, and for CLL patients >60 years chloram-bucil + Rituximab were given. Complete and partial remission of the disease were measured as overall response, while failure of response was defined as stable (did not achieve any remission) or progressive disease. To determine when to start first treatment with early stages disease and to evaluate the disease outcome we followed up patients up to 18 months. Six months post start of treatment; re-evaluation was done for our patients for response assessment according to guidelines of National Cancer Institute-sponsored group [17].

3. Statistical Analysis

Continuous variables were expressed as the mean ± SD & median (range), and the categorical variables were expressed as a number (percentage). Continuous variables were checked for normality by using Shapiro-Wilk test. Mann Whitney U test was used to compare between two groups of non-normally distributed variables. Kruskal Wallis H test was used to compare between more than two groups of normally distributed variables. Percent of categorical variables were compared using the Pearson's Chi-square test or Fisher's exact test when was appropriate. Trend of change in distribution of relative frequencies between ordinal data were compared using Chi-square test for trend. Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of BCL-2/Bax ratio with maximum sensitivity and specificity for prediction of overall response of CLL patients to treatment. Area under Curve (AUROC) was also calculated, criteria to qualify for AUC were as follows: 0.90 - 1 = excellent, 0.80 - 0.90 = good, 0.70 - 0.80 = fair; 0.60 - 0.70 = poor; and 0.50 - 0.6 = fail. The optimal cutoff point was established at point of maximum accuracy. Strength of relationship between time to start first treatment and flow-cytometry markers were determined by computing Spearman's correlation coefficient, (+) sign was indicating direct relationship & (−) sign was indicator for inverse relationship was indicated by (−) sign,

values near to 0 was indicator for weak relationship & values near 1 was indicating strong relationship. All tests were two sided. A significant p-value was <0.05. SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium) was used in performing all statistics.

4. Results

The clinical, laboratory, and outcome of all B-CLL patients are summarized in Table 1. Forty five patients with B-CLL were included; 26 males and 19 females, their age ranged between (38 - 74) years, with a mean value of 56.3 ± 12.3. Twenty patients presented with low stages (0-II) according to Rai staging, and followed up until any indication to start treatment occurred, while 25 patients presented with high stages (III-IV), and began treatment once they diagnosed. MCL-1 was expressed in 60% of B-CLL cases with mean ± SD (60.6 ± 41.98), MCL-1 expression was significantly high in male patients , short lymphocytic doubling time (LDT), and high expression of CD 38 ($p < 0.001$) as shown in Table 2, BCL-2 was expressed in 82.2% of B-CLL cases, with mean ± SD (52.93 + 29.05). There was statistically significant increase in (serum LDH, serum β2M, CD38), low ZAP-70 expression, splenomegaly and higher Rai stage in patients with high expression of BCL-2 compared to those with low expression ($p < 0.001$), Table 3.

4.1. ROC Analysis

To establish the clinical significance of BCL-2/Bax ratio, we calculated the index of test validity for responders to treatment (CR + PR) and non-responders (NR). The best results of sensitivity and specificity were obtained at the cut-off point of 1.6. Table 4 and Figure 2 show ROC curve of BCL-2/Bax ratio as a predictor of overall response of CLL patients to treatment.

There was statistically significant decrease in (HB level, platelet count), and increase in serum LDH, serum β2M, high CD38 expression, low ZAP-70 expression, poor cytogenetic and splenomegaly in patients with high expression of BCL-2/Bax ratio compared to those with low ratio ($p < 0.001$), Table 5.

4.2. The Relation of Apoptosis Regulators and Rai Staging System

We classified our patients according to Rai staging system, and divided them into two groups; low risk group (stage 0-II) and high risk group (stage III–IV). There were significant associations between BCL-2, BCL-2/Bax ratio, and Rai staging system ($p < 0.05$), while MCL-1 expression has no relation to Rai system ($p > 0.05$).

4.3. Response to Treatment Table 6

Thirty-nine patients (86.6%) received chemotherapy during the follow-up period, and demonstrated variable response; 8 patients achieved CR, 12 achieved PR, and 19 were non-responders (NR) to treatment. Six patients did not require starting treatment for CLL, and one patient lost follow-up.

Table 1. Clinical, laboratory, and outcome of all B-CLL patients.

Characteristics Age (year)	No.	(%)	Characteristics Cytogenetic Analysis	No.	(%)
Mean ± SD	56.31	±12.3	Normal	20	(44.4%)
Median (Range)	54	(38 - 74)	del 13	4	(8.9%)
≤60 years	25	(55.6%)	del 11	5	(11.1%)
>60 years	20	(44.4%)	Trisomy 12	6	(13.3%)
Sex			del 17	5	(11.1%)
Male Female		26 (57.8%) 19 (42.2%)	Complex	5	(11.1%)
			CD19 (%)		
Rai classification			Mean ± SD	82.92	±15.67
Stage 0, 1 & 2	20	(44.4%)	Median (Range)	89.10	(32.84 - 97.61)
Stage 3 & 4	25	(55.6%)	CD23 (%)		
Clinical mainfestaion			Mean ± SD	69.40	±27.74
Hepatomegally	22	(48.9%)	Median (Range)	79.27	(0.38 - 95.74)
Lymphadenopathy	31	(68.9%)	CD5/CD20 (%)		
Spleenomegally	33	(73.3%)	Mean ± SD	73.22	±23.52
WBCs ($\times 10^3/mm^3$)			Median (Range)	79.90	(8.41 - 96.02)
Mean ± SD	46.80	±31.71	CD79b (%)		
Median (Range)	31.60	(17.50 - 122.8)	Mean ± SD	57.18	±33.93
$<100 \times 10^3/mm^3$	39	(86.7%)	Median (Range)	66.57	(1.93 - 95.51)
$\geq 100 \times 10^3/mm^3$	6	(13.3%)	FMc7 (%)		
Absolute lymphocytes ($\times 10^3/mm^3$)			Mean ± SD	17.01	±24.89
Mean ± SD	26.27	±16.42	Median (Range)	4.85	(0.06 - 88.44)
Median (Range)	18	(9.10 - 67)	CD22 (%)		
$<30 \times 10^3/mm^3$	33	(73.3%)	Mean ± SD	24.95	±14.39
$\geq 30 \times 10^3/mm^3$	12	(26.7%)	Median (Range)	20.79	(7.12 - 70.69)
Platelet count ($\times 10^3/mm^3$)			CD38 (%)		
Mean ± SD	121.13	±47.20	Mean ± SD	23.79	±25.41
Median (Range)	111	(45 - 205)	Median (Range)	8	(0.30 - 91.59)
$<100 \times 10^3/mm^3$	19	(42.2%)	<30%	28	(62.2%)
$\geq 100 \times 10^3/mm^3$	26	(57.8%)	>30%	17	(37.8%)
Hemoglobin (g/dl)			MCL-1 (%)		
Mean ± SD	10.73	±2.29	Mean ± SD	60.60	±41.98
Median (Range)	10.20	(6.60 - 14.30)	Median (Range)	91.30	(0.60 - 99.70)
<12 g/dl	25	(55.6%)	<25%	18	(40%)
≥12 g/dl	20	(44.4%)	>25%	27	(60%)
LDH (U/L)			BCL-2 (%)		

Continued

Mean ± SD	354	±107.94	Mean ± SD	52.20	±29.24
Median (Range)	340	(190 - 540)	Median (Range)	53.90	(2 - 93.20)
≤350 U/L	23	(51.1%)	<10%	8	(17.8%)
>350 U/L	22	(48.9%)	>10%	37	(82.2%)
B2-microglobulin (mg/L)			ZAP-70		
Mean ± SD	2.91	±0.99	<20%	27	(60%)
Median (Range)	3	(1.40 - 5)	>20%	18	(40%)
<3.5 mg/L	29	(64.4%)	Time to begin ttt (months) for all Pt		
≥3.5 mg/L	16	(35.6%)	Mean ± SD	5.07	±4.23
Coomb's test			Median (Range)	7	(2 - 14)
Negative	38	(84.4%)	Response*		
Positive	7	(15.6%)			
Lymphocytic doubling time (LDT)			No response (NR)	19	(42.2%)
<12 months	21	(46.7%)	Overall response (ORR)	20	(44.5%)
>12 months	24	(53.3%)			

Continuous variables were expressed as mean ± SD & median (range); categorical variables were expressed as number (percentage). *6 patients (13.3%) were not received treatment.

Table 2. Relation between clinicopathological features, flow cytometry markers and MCL-1 expression.

Characteristics	All (N = 45)		MCL-1				*p*-value
			<25% (N = 18)		>25% (N = 27)		
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	56.31	±12.3	52.33	±11.77	58.96	±12.01	0.074•
Median (Range)	54	(38 - 74)	50.50	(38 - 73)	61	(42 - 74)	
≤60 years	25	(55.6%)	13	(52%)	12	(48%)	0.066
>60 years	20	(44.4%)	5	(25%)	15	(75%)	
Sex							
Male	26	(57.8%)	14	(53.8%)	12	(46.2%)	0.027
Female	19	(42.2%)	4	(21.1%)	15	(78.9%)	
Rai classification							
Stage 0, 1 & 2	20	(44.4%)	9	(45%)	11	(55%)	0.540
Stage 3 & 4	25	(55.6%)	9	(36%)	16	(64%)	
Hepatomegally							
Absent	23	(51.1%)	7	(30.4%)	16	(69.6%)	0.181
Present	22	(48.9%)	11	(50%)	11	(50%)	

Continued

Lymphadenopathy							
Absent	14	(31.1%)	7	(50%)	7	(50%)	0.357
Present	31	(68.9%)	11	(35.5%)	20	(64.5%)	
Spleenomegally							
Absent	12	(26.7%)	7	(58.3%)	5	(41.7%)	0.175
Present	33	(73.3%)	11	(33.3%)	22	(66.7%)	
WBCs ($\times 10^3/mm^3$)							
Mean ± SD	46.80	±31.71	49.00	±38.39	45.33	±27.06	0.651•
Median (Range)	31.60	(17.50 - 122.80)	31	(17.50 - 122.80)	37.20	(17.50 - 118.50)	
$<100 \times 10^3/mm^3$	39	(86.7%)	14	(35.9%)	25	(64.1%)	0.199
$\geq 100 \times 10^3/mm^3$	6	(13.3%)	4	(66.7%)	2	(33.3%)	
Absolute lymphocytes ($\times 10^3/mm^3$)							
Mean ± SD	26.27	±16.42	27.60	±21.73	25.39	±12.06	0.487•
Median (Range)	18	(9.10 - 67)	16	(9.10 - 67)	24	(12.50 - 55.20)	
$<30 \times 10^3/mm^3$	33	(73.3%)	13	(39.4%)	20	(60.6%)	1.000
$\geq 30 \times 10^3/mm^3$	12	(26.7%)	5	(41.7%)	7	(58.3%)	
Platelet count ($\times 10^3/mm^3$)							
Mean ± SD	121.13	±47.20	126.77	±45.62	117.37	±48.71	0.378•
Median (Range)	111	(45 - 205)	111.50	(65 - 205)	102	(45 - 200)	
$<100 \times 10^3/mm^3$	19	(42.2%)	6	(31.6%)	13	(68.4%)	0.324
$\geq 100 \times 10^3/mm^3$	26	(57.8%)	12	(46.2%)	14	(53.8%)	
Hemoglobin (g/dl)							
Mean ± SD	10.73	±2.29	10.81	±2.33	10.67	±2.31	0.935•
Median (Range)	10.20	(6.60 - 14.30)	10.40	(7.60 - 14.30)	10.20	(6.60 - 14)	
<12 g/dl	25	(55.6%)	9	(36%)	16	(64%)	0.540
≥12 g/dl	20	(44.4%)	9	(45%)	11	(55%)	
LDH (U/L)							
Mean ± SD	354	±107.94	341.11	±106.70	362.59	±109.91	0.479•
Median (Range)	340	(190 - 540)	340	(200 - 540)	340	(190 - 540)	
≤350 U/L	23	(51.1%)	9	(39.1%)	14	(60.9%)	0.903
>350 U/L	22	(48.9%)	9	(40.9%)	13	(59.1%)	
B2-microglobulin (mg/L)							
Mean ± SD	2.91	±0.99	2.91	±1.02	2.91	±0.99	0.814•
Median (Range)	3	(1.40 - 5)	3.15	(1.40 - 4.40)	3	(1.80 - 5)	
<3.5 mg/L	29	(64.4%)	10	(34.5%)	19	(65.5%)	0.309
≥3.5 mg/L	16	(35.6%)	8	(50%)	8	(50%)	

Continued

Coomb's test							
Negative	38	(84.4%)	16	(42.1%)	22	(57.9%)	0.684
Positive	7	(15.6%)	2	(28.6%)	5	(71.4%)	
Lymphocytic doubling time							
<12 months	21	(46.7%)	12	(57.1%)	9	(42.9%)	0.028
>12 months	24	(53.3%)	6	(25%)	18	(75%)	
Cytogenetic analysis							
Normal	20	(44.4%)	8	(40%)	12	(60%)	0.20
del 13	4	(8.9%)	3	(75%)	1	(25%)	
del 11	5	(11.1%)	3	(60%)	2	(40%)	
Trisomy 12	6	(13.3%)	3	(50%)	3	(50%)	
del 17	5	(11.1%)	0	(0%)	5	(100%)	
Complex	5	(11.1%)	1	(20%)	4	(80%)	
CD38 (%)							
Mean ± SD	23.79	±25.41	19.07	±23.48	26.94	±26.58	0.431•
Median (Range)	8	(0.30 - 91.59)	5.93	(0.30 - 91.57)	32	(0.60 - 91.59)	
<30%	28	(62.2%)	16	(57.1%)	12	(42.9%)	0.003
>30%	17	(37.8%)	2	(11.8%)	15	(88.2%)	
BCL-2 (%)							
Mean ± SD	52.20	±29.24	51.59	±30.40	52.61	±29.03	0.981•
Median (Range)	53.90	(2 - 93.20)	53.90	(3 - 93.20)	48.20	(2 - 91.20)	
<10%	8	(17.8%)	4	(50%)	4	(50%)	0.694§
>10%	37	(82.2%)	14	(37.8%)	23	(62.2%)	
ZAP-70							
<20%	27	(60%)	9	(33.3%)	18	(66.7%)	0.264
>20%	18	(40%)	9	(50%)	9	(50%)	
BCL-2/Bax ratio							
Mean ± SD	2.01	±1.50	1.53	±1.30	2.32	±1.56	0.085•
Median (Range)	1.20	(0.30 - 5)	1	(0.30 - 4.30)	2	(0.50 - 5)	
≤1.6	26	(57.8%)	13	(50%)	13	(50%)	0.109
>1.6	19	(42.2%)	5	(26.3%)	14	(73.7%)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); •Mann Whitney U test; § Chi-square test; $p < 0.05$ is significant.

Table 3. Relation between clinicopathological features, flow cytometry markers and BCL-2 expression.

Characteristics	All (N = 45)		BCL-2				*p*-value
			<10% (N = 8)		>10% (N = 37)		
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	56.31	± 12.3	52.62	± 12.83	57.10	± 12.12	0.298•
Median (Range)	54	(38 - 74)	45.50	(42 - 73)	54	(38 - 74)	
≤60 years	25	(55.6%)	5	(20%)	20	(80%)	0.716
>60 years	20	(44.4%)	3	(15%)	17	(85%)	
Sex							
Male	26	(57.8%)	6	(23.1%)	20	(76.9%)	0.435
Female	19	(42.2%)	2	(10.5%)	17	(89.5%)	
Rai classification							
Stage 0, 1 & 2	20	(44.4%)	7	(35%)	13	(65%)	0.015
Stage 3 & 4	25	(55.6%)	1	(4%)	24	(96%)	
Hepatomegally							
Absent	23	(51.1%)	6	(26.1%)	17	(73.9%)	0.243
Present	22	(48.9%)	2	(9.1%)	20	(90.9%)	
Lymphadenopathy							
Absent	14	(31.1%)	3	(21.4%)	11	(78.6%)	0.689
Present	31	(68.9%)	5	(16.1%)	26	(83.9%)	
Spleenomegally							
Absent	12	(26.7%)	5	(41.7%)	7	(58.3%)	0.022
Present	33	(73.3%)	3	(9.1%)	30	(90.9%)	
WBCs ($\times 10^3/mm^3$)							
Mean ± SD	46.80	±31.71	25.85	±8.49	51.33	±33.11	0.014•
Median (Range)	31.60	(17.50 - 122.80)	25	(17.50 - 38)	36	(17.50 - 122.80)	
$<100 \times 10^3/mm^3$	39	(86.7%)	8	(20.5%)	31	(79.5%)	0.572
$\geq 100 \times 10^3/mm^3$	6	(13.3%)	0	(0%)	6	(100%)	
Absolute lymphocytes ($\times 10^3/mm^3$)							
Mean ± SD	26.27	±16.42	17.01	±6.67	28.28	±17.25	0.085•
Median (Range)	18	(9.10 - 67)	17	(9.10 - 26)	19	(12.50 - 67)	
$<30 \times 10^3/mm^3$	33	(73.3%)	8	(20.5%)	25	(75.8%)	0.087§
$\geq 30 \times 10^3/mm^3$	12	(26.7%)	0	(0%)	12	(100%)	
Platelet count ($\times 10^3/mm^3$)							
Mean ± SD	121.13	±47.20	125	±46.94	120.29	±47.86	0.778•
Median (Range)	111	(45 - 205)	111	(76 - 200)	112	(45 - 205)	
$<100 \times 10^3/mm^3$	19	(42.2%)	2	(10.5%)	17	(89.5%)	0.435§
$\geq 100 \times 10^3/mm^3$	26	(57.8%)	6	(23.1%)	20	(76.9%)	
Hemoglobin (g/dl)							
Mean ± SD	10.73	±2.29	11.73	±2.73	10.51	±2.17	0.191•
Median (Range)	10.20	(6.60 - 14.30)	12.65	(7 - 14.30)	10.10	(6.60 - 14)	
<12 g/dl	25	(55.6%)	2	(8%)	23	(92%)	0.113
≥12 g/dl	20	(44.4%)	6	(30%)	14	(70%)	

Continued

LDH (U/L)							
Mean ± SD	354	±107.94	285	±36.25	368.91	±112.68	0.051•
Median (Range)	340	(190 - 540)	295	(200 - 320)	400	(190 - 540)	
≤350 U/L	23	(51.1%)	8	(34.8%)	15	(65.2%)	0.004
>350 U/L	22	(48.9%)	0	(0%)	22	(100%)	
B2-microglobulin (mg/L)							
Mean ± SD	2.91	±0.99	2.03	±0.44	3.10	±0.98	0.006•
Median (Range)	3	(1.40 - 5)	2	(1.40 - 3)	3	(1.70 - 5)	
<3.5 mg/L	29	(64.4%)	8	(27.6%)	21	(72.4%)	0.037
≥3.5 mg/L	16	(35.6%)	0	(0%)	16	(100%)	
Coomb's test							
Negative	38	(84.4%)	8	(21.1%)	30	(78.9%)	0.321
Positive	7	(15.6%)	0	(0%)	7	(100%)	
Lymphocytic doubling time							
<12 months	21	(46.7%)	7	(33.3%)	14	(66.7%)	0.017
>12 months	24	(53.3%)	1	(4.2%)	23	(95.8%)	
Cytogenetic analysis							
Normal	20	(44.4%)	4	(20%)	16	(80%)	
del 13	4	(8.9%)	2	(50%)	2	(50%)	
del 11	5	(11.1%)	0	(0%)	5	(100%)	0.210
Trisomy 12	6	(13.3%)	2	(33.3%)	4	(66.7%)	
del 17	5	(11.1%)	0	(0%)	5	(100%)	
Complex	5	(11.1%)	0	(0%)	5	(100%)	
CD38 (%)							
Mean ± SD	23.79	±25.41	3.53	±2.38	28.17	±26.03	0.013•
Median (Range)	8	(0.30 - 91.59)	4.41	(0.30 - 5.95)	27.61	(0.60 - 91.59)	
<30%	28	(62.2%)	8	(28.6%)	20	(71.4%)	0.017
>30%	17	(37.8%)	0	(0%)	17	(100%)	
MCL-1 (%)							
Mean ± SD	60.60	±41.98	36.64	±36.07	65.78	±41.79	0.102•
Median (Range)	91.30	(0.60 - 99.70)	31.48	(1.10 - 99.70)	97.47	(0.60 - 99.40)	
<25%	18	(40%)	4	(22.2%)	14	(77.8%)	0.694
>25%	27	(60%)	4	(14.8%)	23	(85.2%)	
ZAP-70							
<20%	27	(60%)	2	(7.4%)	25	(92.6%)	0.045
>20%	18	(40%)	6	(33.3%)	12	(66.7%)	
BCL-2/Bax ratio							
Mean ± SD	2.01	±1.50	0.80	±0.26	2.27	±1.53	0.006•
Median (Range)	1.20	(0.30 - 5)	0.85	(0.30 - 1.10)	2	(0.30 - 5)	
≤1.6	26	(57.8%)	8	(30.8%)	18	(69.2%)	0.014
>1.6	19	(42.2%)	0	(0%)	19	(100%)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); • Mann Whitney U test; § Chi-square test; $p < 0.05$ is significant.

Table 4. BCL-2/Bax ratio as a predictor of overall response (OAR) of CLL patients to treatment; ROC curve analysis.

Cut-off values	SN% (95% CI)	SP% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Accuracy (95% CI)	AUROC (95% CI)	*p*-value
BCL-2/Bax ratio ≤1.6	100% (83.2 - 100)	94.7% (74 - 99.9)	95.2% (76.2 - 99.9)	100% (81.5 - 100)	97.4% (78.7 - 100)	0.982 (0.877 - 1.000)	<0.001

ROC curve: Receiver Operating Characteristic curve; SN: Sensitivity; SP: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; AUROC: Area under Receiver Operating Characteristic curve; 95% CI: 95% Confidence Interval; $p < 0.05$ is significant; Sig.: significance.

Table 5. Relation between clinicopathological features, flow cytometry markersand BCL-2/Bax ratio.

Characteristics	All (N = 45)		BCL-2/Bax ratio				*p*-value
			≤1.6 (N = 26)		>1.6 (N = 19)		
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	56.31	±12.3	56.19	±12.92	56.47	±11.55	0.927•
Median (Range)	54	(38 - 74)	53.50	(38 - 74)	54	(42 - 74)	
≤60 years	25	(55.6%)	15	(60%)	10	(55%)	0.73
>60 years	20	(44.4%)	11	(55%)	9	(45%)	
Sex							
Male	26	(57.8%)	16	(61.5%)	10	(38.5%)	0.550
Female	19	(42.2%)	10	(52.6%)		9(47.4%)	
Rai classification							
Stages 0, 1 & 2	20	(44.4%)	17	(85%)	3	(15%)	0.001
Stages 3 & 4	25	(55.6%)	9	(36%)	16	(64%)	
Hepatomegally							
Absent	23	(51.1%)	13	(56.5%)	10	(43.5%)	0.862
Present	22	(48.9%)	13	(59.1%)	9	(40.9%)	
Lymphadenopathy							
Absent	14	(31.1%)	9	(64.3%)	5	(35.7%)	0.553
Present	31	(68.9%)	17	(54.8%)	14	(45.2%)	
Spleenomegally							
Absent	12	(26.7%)	11	(91.7%)	1	(8.3%)	0.006
Present	33	(73.3%)	15	(45.5%)	18	(54.5%)	
WBCs ($\times 10^3/mm^3$)							
Mean ± SD	46.80	±31.71	47.90	±37.22	45.30	±23	0.401•
Median (Range)	31.60	(17.50 - 122.80)	33.25	(17.50 - 122.80)	31.60	(17.50 - 112.50)	
$<100 \times 10^3/mm^3$	39	(86.7%)	21	(53.8%)	18	(46.2%)	0.222
$\geq 100 \times 10^3/mm^3$	6	(13.3%)	5	(83.3%)	1	(16.7%)	
Absolute lymphocytes ($\times 10^3/mm^3$)							
Mean ± SD	26.27	±16.42	26.80	±17.42	25.55	±15.37	0.881•
Median (Range)	18	(9.10 - 67)	18.65	(9.10 - 67)	17.30	(13 - 67)	
$<30 \times 10^3/mm^3$	33	(73.3%)	19	(57.6%)	14	(42.4%)	0.964
$\geq 30 \times 10^3/mm^3$	12	(26.7%)	7	(58.3%)	5	(41.7%)	
Platelet count ($\times 10^3/mm^3$)							
Mean ± SD	121.13	±47.20	134.69	±48.89	102.57	±38.69	0.035•
Median (Range)	111	(45 - 205)	125.50	(45 - 205)	90	(49 - 181)	
$<100 \times 10^3/mm^3$	19	(42.2%)	7	(36.8%)	12	(63.2%)	0.015
$\geq 100 \times 10^3/mm^3$	26	(57.8%)	19	(73.1%)	7	(26.9%)	

Continued

Hemoglobin (g/dl)							
Mean ± SD	10.73	±2.29	11.56	±2.16	9.58	±2.01	0.009•
Median (Range)	10.20	(6.60 - 14.30)	12.50	(7 - 14.30)	9.10	(6.60 - 14)	
<12 g/dl	25	(55.6%)	9	(36%)	16	(64%)	0.001
≥12 g/dl	20	(44.4%)	17	(85%)	3	(15%)	
LDH (U/L)							
Mean ± SD	354	±107.94	305.76	±89.94	420	±96.37	0.001•
Median (Range)	340	(190 - 540)	300	(190 - 500)	440	(210 - 540)	
≤350 U/L	23	(51.1%)	19	(82.6%)	4	(17.4%)	0.001
>350 U/L	22	(48.9%)	7	(31.8%)	15	(68.2%)	
B2-microglobulin (mg/L)							
Mean ± SD	2.91	±0.99	2.67	±0.97	3.25	±0.95	0.046•
Median (Range)	3	(1.40 - 5)	2	(1.40 - 4.40)	3.30	(2 - 5)	
<3.5 mg/L	29	(64.4%)	18	(62.1%)	11	(37.9%)	0.433
≥3.5 mg/L	16	(35.6%)	8	(50%)	8	(50%)	
Coomb's test							
Negative	38	(84.4%)	24	(63.2%)	14	(36.8%)	0.114
Positive	7	(15.6%)	2	(28.6%)	5	(71.4%)	
Lymphocytic doubling time							
<12 months	21	(46.7%)	18	(85.7%)	3	(14.3%)	<0.001
>12 months	24	(53.3%)	8	(33.3%)	16	(66.7%)	
Cytogenetic analysis							
Normal	20	(44.4%)	15	(75%)	5	(25%)	
del 13	4	(8.9%)	3	(75%)	1	(25%)	
del 11	5	(11.1%)	1	(20%)	4	(80%)	0.001
Trisomy 12	6	(13.3%)	6	(100%)	0	(0%)	
del 17	5	(11.1%)	1	(20%)	4	(80%)	
Complex	5	(11.1%)	0	(0%)	5	(100%)	
CD38 (%)							
Mean ± SD	23.79	±25.41	11.76	±13.31	40.26	±28.94	<0.001•
Median (Range)	8	(0.30 - 91.59)	4.90	(0.30 - 42)	38	(0.72 - 91.59)	
<30%	28	(62.2%)	23	(82.1%)	5	(17.9%)	<0.001
>30%	17	(37.8%)	3	(17.6%)	14	(82.4%)	
MCL-1 (%)							
Mean ± SD	60.60	±41.98	49.14	±42.41	76.27	±36.89	0.087•
Median (Range)	91.30	(0.60 - 99.70)	38.03	(0.60 - 99.70)	98	(9.70 - 99.20)	
<25%	18	(40%)	13	(72.2%)	5	(27.8%)	0.109
>25%	27	(60%)	13	(48.1%)	14	(51.9%)	
BCL-2 (%)							
Mean ± SD	52.20	±29.24	46.47	±34.34	60.04	±18.45	0.223•
Median (Range)	53.90	(2 - 93.20)	48.20	(2 - 93.20)	66.20	(35.70 - 93.20)	
<10%	8	(17.8%)	8	(100%)	0	(0%)	0.014
>10%	37	(82.2%)	18	(48.6%)	19	(51.4%)	
ZAB-70							
<20%	27	(60%)	11	(40.7%)	16	(59.3%)	0.005
>20%	18	(40%)	15	(83.3%)	3	(16.7%)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); • Mann Whitney U test; § Chi-square test; $p < 0.05$ is significant.

Table 6. Relation between response and clinicopathological features, flow cytometry markers.

Characteristics	All (N = 39)		Response				*p*-value
			No response (N = 19)		OAR (N = 20)		
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	57.79	±12.01	56.57	±11.45	58.95	±12.71	0.473•
Median (Range)	54	(39 - 74)	54	(42 - 74)	59	(39 - 74)	
≤60 years	20	(51.3%)	10	(50%)	10	(50%)	0.869§
>60 years	19	(48.7%)	9	(47.4%)	10	(52.6%)	
Sex							
Male	23	(59%)	10	(43.5%)	13	(56.5%)	0.433§
Female	16	(41%)	9	(56.3%)	7	(43.8%)	
Rai classification							
Stages 0, 1 & 2	16	(41%)	3	(18.8%)	13	(81.3%)	0.002§
Stages 3 & 4	23	(59%)	16	(69.6%)	7	(30.4%)	
Hepatomegally							
Absent	21	(53.8%)	11	(52.4%)	10	(47.6%)	0.621§
Present	18	(46.2%)	8	(44.4%)	10	(55.6%)	
Lymphadenopathy							
Absent	13	(33.3%)	5	(38.5%)	8	(61.5%)	0.365§
Present	26	(66.6%)	14	(53.8%)	12	(46.2%)	
Spleenomegally							
Absent	9	(23.1%)	1	(11.1%)	8	(88.9%)	0.020§
Present	30	(76.9%)	18	(60%)	12	(40%)	
WBCs ($\times 10^3/mm^3$)							
Mean ± SD	49.44	±33.21	47.46	±23.50	51.32	±40.93	0.407•
Median (Range)	36	(17.50 - 122.80)	43	(17.50 - 112.50)	32.35	(17.50 - 122.80)	
$<100 \times 10^3/mm^3$	33	(84.6%)	18	(54.5%)	15	(45.5%)	0.182§
$\geq 100 \times 10^3/mm^3$	6	(15.4%)	1	(16.7%)	5	(83.3%)	
Absolute lymphocytes ($\times 10^3/mm^3$)							
Mean ± SD	27.72	±17.09	26.61	±15.29	28.79	±18.97	0.855•
Median (Range)	19.30	(9.10 - 67)	19	(13 - 67)	21.15	(9.10 - 67)	
$<30 \times 10^3/mm^3$	27	(69.2%)	13	(48.1%)	14	(51.9%)	0.915§
$\geq 30 \times 10^3/mm^3$	12	(30.8%)	6	(50%)	6	(50%)	
Platelet count ($\times 10^3/mm^3$)							
Mean ± SD	121.61	±48.49	97.68	±33.79	144.35	±50.05	0.004•
Median (Range)	112	(45 - 205)	89	(49 - 180)	159.50	(45 - 205)	

Continued

<100 × 10^3/mm^3	17	(43.6%)	13	(76.5%)	4	(23.5%)	0.002§
≥100 × 10^3/mm^3	22	(56.4%)	6	(27.3%)	16	(72.7%)	
Hemoglobin (g/dl)							
Mean ± SD	10.79	±2.25	9.59	±2.01	11.93	±1.87	0.002•
Median (Range)	10.20	(6.60 - 14.30)	9.10	(6.60 - 14)	12.95	(7.70 - 14.30)	
<11 g/dl	22	(56.4%)	16	(72.7%)	6	(27.3%)	0.001§
≥11 g/dl	17	(43.6%)	3	(17.6%)	14	(82.4%)	
LDH (U/L)							
Mean ± SD	363.84	±108.13	419.47	±96.29	311	±96.56	0.002•
Median (Range)	380	(190 - 540)	430	(210 - 540)	300	(190 - 500)	
≤350 U/L	18	(46.2%)	4	(22.2%)	14	(77.8%)	0.002§
>350 U/L	21	(53.8%)	15	(71.4%)	6	(28.6%)	
B2-microglobulin (mg/L)							
Mean ± SD	3.01	±0.97	3.25	±0.95	2.78	±0.96	0.130•
Median (Range)	3	(1.80 - 5)	3.30	(2 - 5)	2.05	(1.80 - 4.40)	
<3.5 mg/L	24	(61.5%)	11	(45.8%)	13	(54.2%)	0.648§
≥3.5 mg/L	15	(38.5%)	8	(53.3%)	7	(46.7%)	
Coomb's test							
Negative	32	(82.1%)	13	(40.6%)	19	(59.4%)	0.044•
Positive	7	(17.9%)	6	(85.7%)	1	(14.3%)	
Lymphocytic doubling time							
<12 months	18	(46.2%)	3	(16.7%)	15	(83.3%)	<0.001§
>12 months	21	(53.8%)	16	(76.2%)	5	(23.8%)	
Cytogenetic analysis							
Normal	17	(43.6%)	5	(29.4%)	12	(70.6%)	0.002§
del 13	3	(7.7%)	1	(33.3%)	2	(66.7%)	
del 11	4	(10.3%)	3	(75%)	1	(25%)	
Trisomy 12	5	(12.8%)	0	(0%)	5	(100%)	
del 17	5	(12.8%)	5	(100%)	0	(0%)	
Complex	5	(12.8%)	5	(100%)	0	(0%)	
CD38 (%)							
Mean ± SD	25.94	±26.21	41.69	±27.74	10.98	±12.70	<0.001•
Median (Range)	22	(0.30 - 91.59)	38	(0.72 - 91.59)	4.59	(0.30 - 42)	
<30%	23	(59%)	4	(17.4%)	19	(82.6%)	<0.001§
>30%	16	(41%)	15	(93.8%)	1	(6.3%)	
ZAP-70							
<20%	24	(61.5%)	16	(66.7%)	8	(33.3%)	0.005§
>20%	15	(38.5%)	3	(20%)	12	(80%)	

Continued

MCL-1 (%)							
Mean ± SD	64.98	±41.21	80.16	±34.13	50.55	±42.94	0.177•
Median (Range)	96.60	(0.60 - 99.70)	98	(9.70 - 99.20)	38.03	(0.60 - 99.70)	
<25%	14	(35.9%)	4	(28.6%)	10	(71.4%)	0.060§
>25%	25	(64.1%)	15	(60%)	10	(40%)	
BCL-2 (%)							
Mean ± SD	56.72	±27.64	59.74	±18.60	53.86	±34.39	0.855•
Median (Range)	66.10	(2 - 93.20)	66.20	(35.70 - 93.20)	61.45	(2 - 93.20)	
<10%	5	(12.8%)	0	(0%)	5	(100%)	0.047§
>10%	34	(87.2%)	19	(55.9%)	15	(44.1%)	
BCL-2/Bax ratio							
Mean ± SD	2.16	±1.54	3.50	±1.10	0.89	±0.32	<0.001•
Median (Range)	1.50	(0.30 - 5)	3.70	(1 - 5)	0.95	(0.30 - 1.60)	
≤1.6	21	(53.8%)	1	(4.8%)	20	(95.2%)	<0.001§
>1.6	18	(46.2%)	18	(100%)	0	(0%)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); •Mann Whitney U test; § Chi-square test; $p < 0.05$ is significant.

Although MCL-1 expression was not significantly affect the treatment response ($p = 0.060$), better overall response was associated with low MCL-1 expression, as 10/14 of those with low MCL-1developed objective response to treatment (CR + PR).

Low BCL-2 expression was significantly associated with achieving treatment response but when CR and PR were calculated together ($p = 0.047$), as all patients with low expression of BCL-2 were achieving overall response, and the 19 non responding patients were having high BCL-2 expression. Also low BCL-2/Bax ratio was significantly associated with better treatment response (CR+PR) ($p = 0.001$); among the 39 patients who started treatment at diagnosis, responding patients had statistically significant lower BCL-2/Bax ratio mean than non-responding patients (0.89 ± 0.32 vs. 3.5 ± 1.1, respectively; $p < 0.001$), and none of the patients in the group with high BCL-2/Bax ratio achieving any response, while 95% of responding patients had low BCL-2/Bax ratio.

4.4. Time to Start Treatment

Our patients were grouped according to Rai staging system, to two categories; low risk group (stage 0-II) and high risk (stage III–IV). The low risk group (20 patients) was followed up to detect time to start treatment ,and to determine factors that influence that time, so 20 patients were followed for up to 18 months; 6 of them didn't need to start treatment. The time to start chemotherapy treatment ranging from 2 to 14 months, we found a significant difference between patients ≤60 years and those >60 years as regard time to start treatment ($p = 0.026$). Patients with high BCL-2 expression and those with high BCL-2/Bax ratio experienced shorter time to start treatment, but that was not statistically significant ($p > 0.05$) Table 7, Figure 3 and Figure 4.

Table 7. Relation between time to start treatment in low risk group CLL patients and prognostic factors.

Characteristics	All (N = 20)		Time to start treatment (months)				*p*-value
	No.	(%)	Mean	±SD	Median	(Range)	
Age							
≤60 years	12	(60%)	11.16	±3.37	12.50	(4 - 14)	0.026•
>60 years	8	(40%)	7.75	±3.28	7.50	(2 - 12)	
Sex							
Male	14	(70%)	9.85	±4.14	11.5	(2 - 14)	0.919*
Female	6	(30%)	9.66	±2.58	9	(7 - 14)	
Hepatomegally							
Absent	11	(55%)	9.63	±3.58	11	(4 - 14)	0.833*
Present	9	(45%)	10	±4	10	(2 - 14)	
Lymphadenopathy							
Absent	7	(35%)	10.14	±4.18	11	(2 - 14)	0.769*
Present	13	(65%)	9.61	±3.54	8	(4 - 14)	
Spleenomegally							
Absent	11	(55%)	10.45	±3.38	12	(4 - 14)	0.394*
Present	9	(45%)	9	±4.06	10	(2 - 14)	
WBCs							
$<100 \times 10^3/mm^3$	15	(75%)	10.46	±3.52	11	(4 - 14)	0.157•
$\geq 100 \times 10^3/mm^3$	5	(25%)	7.80	±3.76	8	(2 - 12)	
Absolute lymphocytes							
$<30 \times 10^3/mm^3$	13	(65%)	11.15	±3.23	12	(4 - 14)	0.018•
$\geq 30 \times 10^3/mm^3$	7	(35%)	7.28	±3.25	7	(2 - 12)	
Lymphocytic doubling time							
<12 months	14	(70%)	10.14	±3.63	10.50	(2 - 14)	0.539*
>12 months	6	(30%)	9	±4	9.50	(4 - 14)	
Cytogenetic analysis							
Normal	12	(60%)	9.58	±3.50	9.50	(4 - 14)	
del 13	3	(15%)	10	±3.46	8	(8 - 14)	0.946‡
del 11	1	(5%)	11				
Trisomy 12	4	(20%)	10	±5.65	12	(2 - 14)	
CD38							
<30%	17	(85%)	10.17	±3.35	11	(2 - 14)	0.288*
>30%	3	(15%)	7.66	±5.50	5	(4 - 14)	
MCL-1							
<25%	9	(45%)	10.66	±4.03	12	(2 - 14)	0.233•
>25%	11	(55%)	9.09	±3.38	8	(4 - 14)	
BCL-2							
<10%	7	(35%)	11.71	±2.75	13	(8 - 14)	0.077•
>10%	13	(65%)	8.76	±3.78	8	(2 - 14)	
ZAB-70							
<20%	6	(30%)	8.50	±3.88	8	(4 - 14)	0.314*
>20%	14	(70%)	10.35	±3.58	11	(2 - 14)	
BCL-2/Bax ratio							
≤1.6	17	(85%)	10.35	±3.69	11	(2 - 14)	0.077•
>1.6	3	(15%)	6.66	±1.52	7	(5 - 8)	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range); *Independent samples Student's t-test; •Mann Whitney U test; ‡Kraskall Wallis H test; $p < 0.05$ is significant.

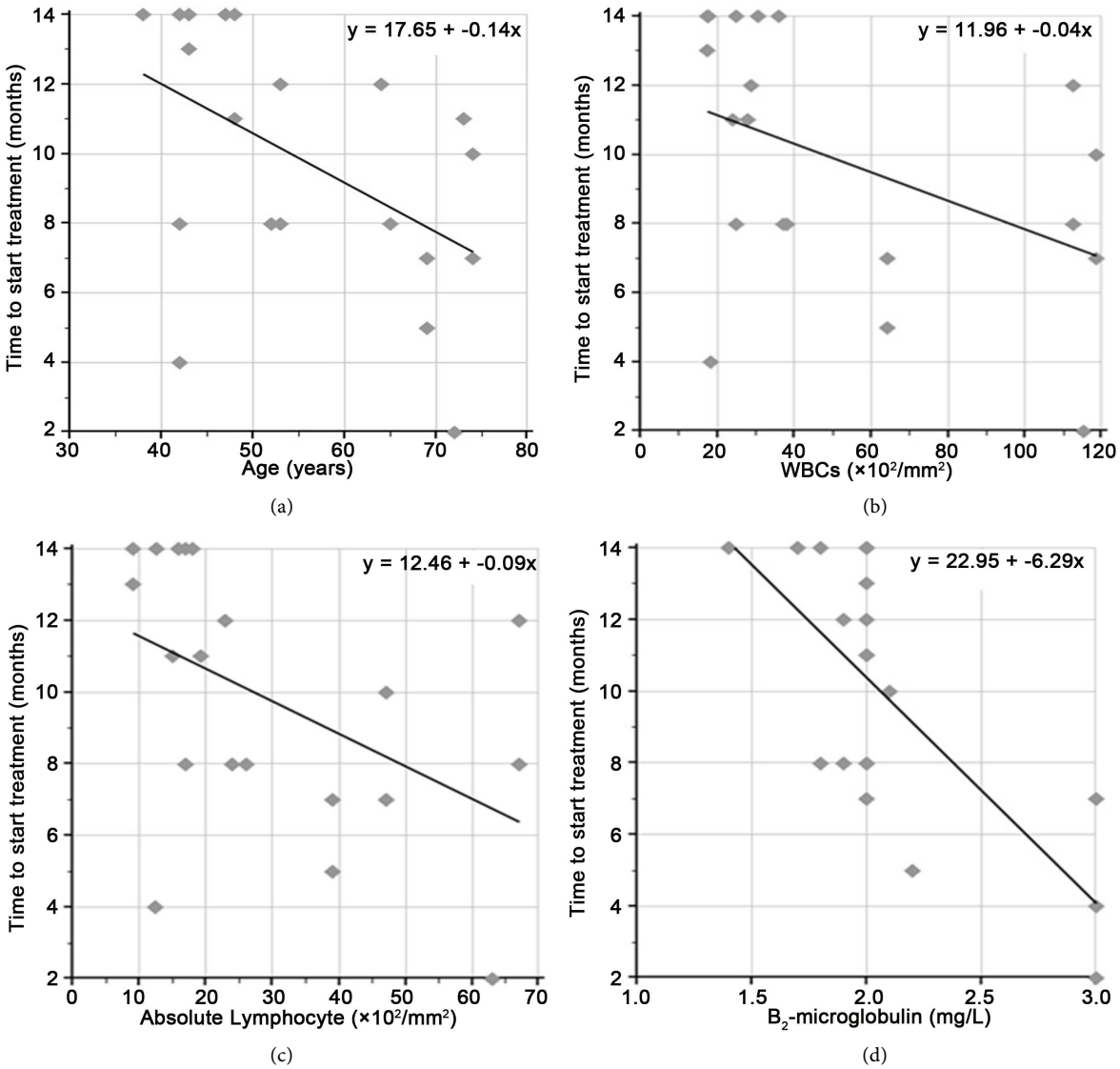
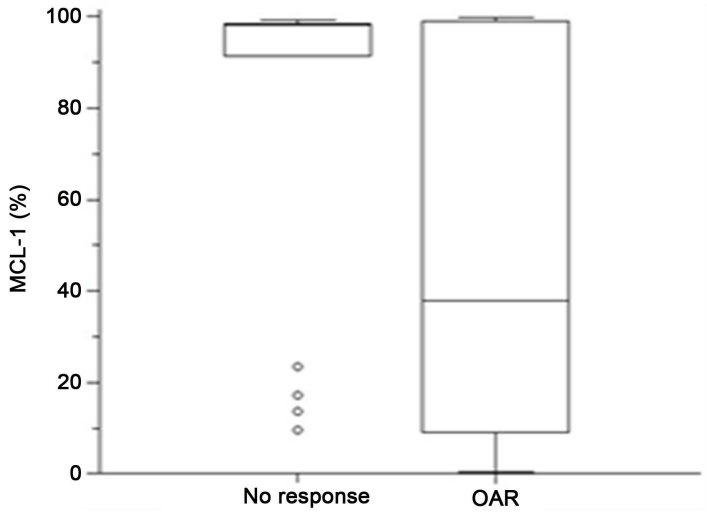


Figure 3. Scatter plot with regression line shows indirect correlation between (a) Age (years); (b) WBCs (×10³/mm³); (c) Absolute lymphocyte count (×10³/mm³); (d) B2-microglobulin (mg/L) and time to start first treatment (months).

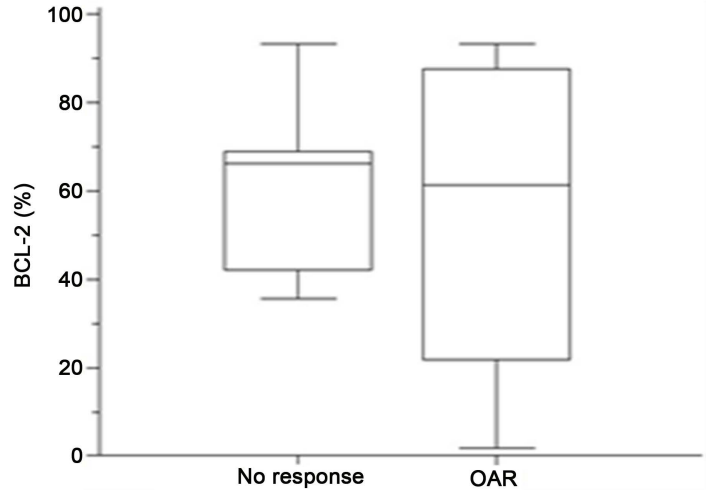
Also we found a significant indirect correlation between age, WBCS count, Absolute lymphocyte count, B2-microglobulin and time to start first treatment (months) Table 8.

5. Discussion

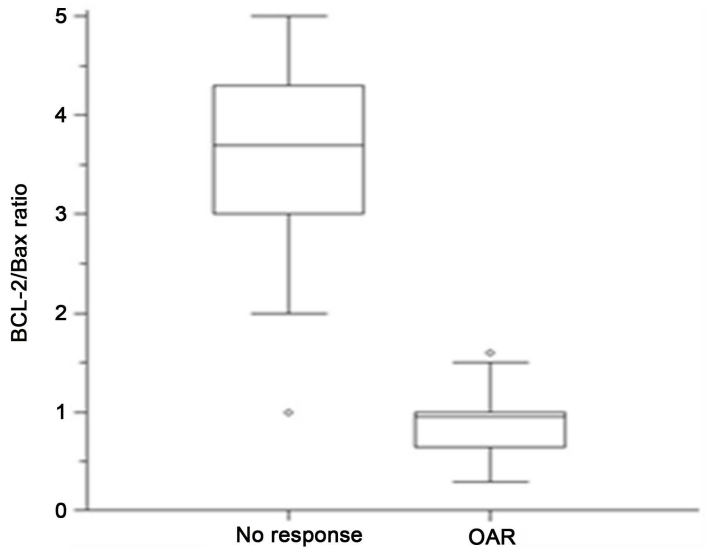
Nowadays with the development of novel treatment options it necessitate the identification of patients ,with unfavorable prognostic features ,who are more liable for early progression and who would gain the most benefit from early interference with targeted treatment. For this reason, identification of prognostic factors of CLL is the interest of all researchers [18]. As known BCL-2 is working as an anti-apoptosis and it is an integral inner mitochondrial membrane protein; its over-expression prevent the apoptotic death of a pro-B-lymphocyte cell line. Thus, BCL-2 is unique among proto-oncogene, being localized in mitochondria



(a)



(b)



(c)

Figure 4. Box plot shows comparison between non-responder and responder as regard (a) MCL-1 (%); (b) BCL-2 (%) and (c) BCL-2/Bax ratio.

Table 8. Correlation between time to start treatment (months) and prognostic markers.

Variables	Time to start first treatment (months)	
	r	*p*-value
Age (years)	−0.498	0.025
WBCs ($\times 10^3/mm^3$)	−0.511	0.021
Absolute lymphocytes ($\times 10^3/mm^3$)	−0.507	0.023
Platelet count ($\times 10^3/mm^3$)	−0.274	0.242
Hemoglobin (g/dl)	−0.203	0.392
LDH (U/L)	−0.010	0.967
B2-microglobulin (mg/L)	−0.665	0.001
CD38 (%)	−0.209	0.376
MCL-1 (%)	−0.239	0.310
BCL-2 (%)	−0.305	0.191
BCL-2/Bax ratio	−0.388	0.091

r: Spearman's rank correlation coefficient; $p < 0.05$ is significant.

and interfering with apoptosis independent of cell division promotion [19]. Although the exact mechanism of BCL-2 action is not known, high expression of BCL-2 correlates with increased cell survival through reduction of apoptosis by interfering sequences that lead to apoptosis [20], We have known several members of anti-apoptotic and pro-apoptotic BCL-2 family, but MCL-1 is the most significant anti-apoptotic protein associated with normal as well as malignant B lymphocytes, it is essential during both lymphoid development and maintenance of mature T and B lymphocytes [21].

Our study showed that 27 patients (60%) were positive MCL-1, while Anurag Saxena *et al.* [15] found that 72% of patients were positive MCL-1. No significance between MCL-1 and Rai classification was found, and this in accordance with Anurag Saxena *et al.* [15], who reported that MCL-1 did not has significant association with Rai stage. This in contrary to Pepper *et al.* [6], who reported that, MCL-1 expression was significantly correlated with stage of disease ($p < 0.001$), and this is may be explained partially by larger sample size (185 patients). MCL-1 showed positive significant correlation with expression of CD38 in our study ($p = 0.002$), and this in agreement with Pepper *et al.* [6] ($p < 0.001$).

In our study an association between low MCL-1 levels and ability to gain overall response (10/14) to treatment was detected, in Anurag Saxena study [15], and in Kitada's report [22], all patients with CR had low MCL-1 levels. Also Kitada's reported that MCL-1 is the only anti-apoptotic protein which was identified to be associated with in vitro resistance to chlormabucil and fludarabine and significantly lower CR rates in patients with CLL [22]. MCL-1upregulation in CLL patients may be related to certain growth factors e.g., interleukin-4 (IL-4), and IL-13 [23], or alternation in structure of MCL-1 gene [24], that lead to MCL-1 protein persistent elevations in those patients. Marschitz *et al.* [25], mentioned that BCL-2 strong expression is a constant feature of CLL cells , Srinivas *et al.* [16] pointed that the cut-off level was defined as more than 10%, In our study, 37 cases had BCL-2+ cells > 10% and this represented 82.2% of the studied cases , Shinichi *et al.* [26], reported that the levels of BCL-2 protein expres-

sion were 60% of B-CLL patients, While in study by Lazaridou *et al.* [27], the levels of BCL-2 protein expression were 76.3% of the CLL patients, and Mendez *et al.* [28], showed that the levels of BCL-2 protein expression were 77% of B-CLLs. This difference may be due to late diagnosis, or high Rai classification in our patients. Marschitz *et al.* [25], found higher levels of BCL-2 in patients with progressive disease. In our study, we found positive significant correlation between Rai staging and splenomegaly with BCL-2 expression levels ($p < 0.005$). Marschitz *et al.* [25]), and Anurag *et al.* [15] reported that BCL-2 expression levels were not correlated with Rai stages of disease this is, possibly due to different methods of measurement (e.g. western blot technique). In our study, high BCL-2 expression was correlated with short lymphocytic doubling time, high LDH, high serum β2M, high CD38 expression and low ZAP-70 expression. As regard the treatment response, we found low BCL-2 expression was significantly associated with better treatment response only when CR and PR were considered together ($p = 0.047$), and this in agreement with Schimmer *et al.* [29], who pointed that, aberrant expression of BCL-2 was associated with poor response to chemotherapy and decreased overall survival.

Regard the clinical impact of BCL-2/Bax ratio in the present study; as independent prognostic factor in CLL patients, we detected that a higher BCL-2/Bax ratio strongly correlated with some unfavorable clinical presentations like low HB, low platelets , high LDH and splenomegaly, also significant associations detected between high BCL-2/Bax ratio and indicators of higher tumor burden (B2M, and LDT, Rai stages) and other prognostic markers such CD38, and cytogenetics, and CD38 overexpression is well known to has adverse prognostic effect in CLL [30] [31], and there were significant correlations between BCL-2, MCL-1, and BCL-2/Bax ratio with CD38 expression, so estimation of BCL-2, MCL-1 and BCL-2/Bax ratio expressions in CLL cases could be used as predictors of bad prognosis. Kitada *et al.* [22] mentioned that in his result increased BCL-2/Bax ratio was associated with high total leucocyte count, also Anurag Saxena *et al.* [15], noted that in BCL-2/Bax positive patients the LDT was significantly longer, which suggests a significant correlation between this ratio and high proliferation. In our study, low BCL-2/Bax ratio showed a statistically significant association with treatment response when CR + PR were calculated together, and this in agreement with Anurag Saxena *et al.* [15], who noted BCL-2/Bax ratio was significantly associated with treatment response (0.89 ± 0.53 [CR + PR] vs. 3.38 ± 4.47 [NR] ($p = 0.011$)) with others results it there was an association between a high BCL-2/Bax ratio and treatment resistance [11] [12] [13]. Correlations between BCL-2/Bax ratios and chlorambucil was demonstrated in some in vitro studies [32], with fludarabine-induced apoptosis [33] [34], and also with steroid-induced apoptosis [35]. In CLL patients high BCL-2/Bax ratio may reflect resistant clones [32], however, this point still with some controversy in literature, as some investigators have not found any correlation between in vitro or in vivo sensitivity to chlorambucil, fludarabine, and BCL-2, BAX , and their ratio [18] [22]. Also, Zaja *et al.*, failed in his study to show any correlation

between BCL-2 and treatment response [36]. These controversies may be explained by variability in the comparing and measuring protein levels by using different standard, also the samples collection time in relation to therapy timing may play role in these variabilities.

6. Conclusion

By using a flow cytometric method, we detected the prognostic power of MCL-1, BCL-2, and BCL-2/Bax ratio, which is an easy method used in routine laboratory practice. Also we defined the correlations of these proteins expressions with chemo-resistance and clinical outcome in CLL patients. So MCL-1, BCL-2, and BCL-2/Bax ratio can be used to determine CLL cases that can be targeted by new BCL-2 inhibitors therapy.

Conflict of Interest

The authors have declared no conflict of interest.

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