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ZAP-70 and CD38 Expression in Egyptian CLL

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ABSTRACT

Introduction: Following gene expression profiling which compared the two well established prognostic subsets (ZAP-70 and CD38), with unmutated and mutated IgV_H, ZAP-70 has emerged as the most promising surrogate marker for the IgV_H mutation status. CD38 expression has been also suggested as a surrogate marker for the IgV_H mutation status. However, in subsequent studies, researchers could clearly demonstrate the clinical value of CD38 as a prognostic marker with some degree of correlation to IgV_H mutation.

Aim of the Work: The aim of this study was to investigate the role of ZAP-70 and CD38 as predictors of disease progression in chronic lymphocytic leukaemia and their relations to the other prognostic markers.

Patients and Methods: This study included 50 CLL patients as well as 10 age and sex matched normal volunteers as a control group. All patients were subjected to Immunophenotyping using flowcytometer, Partec III to confirm the diagnosis of CLL with a wide panel of monoclonal antibodies. After appropriate lymphocyte gating, cytoplasmic ZAP-70 and surface CD38 expression were determined in (CD19+, CD5+) B cells. Results were correlated to other known prognosting markers of CLL.

Resuts: No significant association was found between ZAP-70% or CD38% expression and Rai staging at diagnosis; however, a significant positive association was found between CD38 measured as mean fluorescent index (MFI) and Rai staging at diagnosis (p=0.019).

A significant positive association was found between ZAP-70% expression and the non-responders group (those with stable or progressive disease after receiving 3-6 cycles of chemotherapy) and the bone marrow diffuse pattern of infiltration (p<0.001, 0.002 respectively). Also, a significant positive relation was found between ZAP-70% expression and P53% expression (p=0.005).

A significant increase in serum levels of LDH and $ß_2$M in ZAP-70 positive groups as compared to ZAP-70 negative group was detected (p=0.049 and 0.007 respectively).

CD38, either expressed as a percentage or as MFI, showed a significant positive association with the non-responders group (p=0.034, 0.006 respectively) and no significant association with BMB infiltration pattern.

A significant increase in serum levels of B2M in CD38% positive group as compared to CD38 negative group was encountered (p=0.045). A non-significant difference was found with LDH and P53 in CD38 positive as compared to CD38 negative groups. However CD38-MFI showed a significant positive relation to both LDH and B_2M (p=0.03 and 0.05 respectively).

By using the combined expressions of both markers, there was a significant association in concordant positive group (ZAP-70+/CD38+) with poor initial response to chemotherapy (p<0.001) and diffuse BMB infiltration pattern (p=0.015). Also, there was a significant positive relation with the increase in P53% expression (p<0.05), LDH (p=0.029) and $ß_2$-M (p<0.001).

A significant positive association was found between ZAP-70% expression as well as the combined expression of both ZAP-70 and CD38 and time to disease progression (TDP) (p=0.025 and <0.001) respectively.

A significant negative association was found between overall survival (O.S) and ZAP-70% expression as well as the combined expression of both ZAP-70 and CD38 (p=0.029 and 0.0312 respectively).

Conclusion: ZAP-70 is one of the most important prognostic markers in CLL, it appeared to be more predictive of disease progression and poor outcome than CD38 expression. Semi quantification of the CD38 antigen by flowcytometry greatly improves the prognostic value of the percentage expression. The combination of ZAP-70 and CD38 increases the prognostic power of either of the two factors.

Key Words: *ZAP-70 – CD38 – CLL.*

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disorder characterized by a variable clinical course **[1]**. Some patients have an aggressive disease requiring early therapy, whereas other patients exhibit a more stable, indolent disease with no benefit from palliative chemotherapy **[2]**. In a continual effort to identify

patients with poor prognosis and to facilitate the clinical management of B-CLL, several prognostic markers have been identified during the last two decades **[3]**.

At first, clinical staging systems based on leukemia cell burden were developed. Those systems have delineated the clinical presentation and natural history of B-CLL and have allowed predicting survival and treatment requirements **[4,5]**. However, the Rai and Binet staging systems lack the ability to distinguish prospectively patients with early stage B-CLL that will rapidly progress to aggressive disease from patients destined to remain in early stage for a long time **[1]**. Therefore, other parameters related to the genetics and biology of B-CLL, such as genomic aberrations and immunoglobulin variable heavy chain (IgV_H) mutation status, are increasingly used for prediction of disease prognosis **[3]**.

In landmark studies, it has been shown that survival probability in B-CLL is associated with mutational status of IgV_H genes **[6,7]**. Currently, IgV_H gene mutation status is considered as one of the most powerful prognostic factors, where B-CLL cases with unmutated IgV_H genes are characterized by an unfavorable clinical outcome **[1,3]**.

However, IgV_H mutation analysis is based on DNA sequencing, which is technically demanding and not widely available for routine clinical use. Thus, easily determined surrogate markers for IgV_H mutations are required. DNA microarray studies have shown that B-CLL cells with unmutated (IgV_H) genes can be distinguished from those with mutated IgV_H genes by the differential expression of a small number of genes, one of which encodes the 70-kDa zeta associated protein (ZAP-70) **[8,9]**.

ZAP-70, a member of the Syk-ZAP-70 protein tyrosine kinase family, is a key signaling molecule for T lymphocytes and natural killer cells. While ZAP-70 is not expressed in normal B lymphocytes, it is associated with increased intracellular signaling via the immunoglobulin receptor in B-CLL cells **[10,11]**.

Considering two published studies, **[12,13]**, ZAP-70 is the most promising surrogate marker for the IgV_H mutation status. In contrast to the technically demanding IgV_H analysis, ZAP-70 protein expression is conveniently measured by flowcytometry **[14,15]**.

Initially, CD38 expression also has been conveniently considered as a surrogate marker for the two important IgV_H mutated and unmutated subgroups of B-CLL **[16]**. CD38 is a type II transmembrane glycoprotein that acts as a complex ecto-enzyme and receptor molecule with signaling functions in B-CLL cells **[17]**.

However, while a plethora of subsequent studies could clearly demonstrate the clinical value of CD38 as a prognostic marker with some degree of correlation to IgV_H mutation status, both ZAP-70 and CD 38 are regarded as independent prognostic variables in B-CLL **[18]**.

PATIENTS AND METHODS

(A) Patients:

The present study was carried out in the Clinical Pathology Department of the National Cancer Institute (NCI), Cairo University during the period between November 2004 and November 2006.

The patients were selected from the outpatient clinic of the Medical Oncology Department. Fifty patients with chronic lymphocytic leukemia (CLL) were included in this study, forty males (80%) and ten females (20%) and their ages ranged from 37 to 80 years. Ten age and sex-matched normal volunteers were used as a control group.

The diagnosis of CLL was based on the criteria established by the International Work Shop on CLL and the National Cancer Institute-Sponsored Working Group Guidelines for CLL (NCI-WG) **[19]**. All cases were staged according to modified Rai stage system **[20]**. Twenty-six patients (52%) were at low risk Rai stages (0, I, II), 13 patients (26%) were at intermediate Rai stage (III) and 11 patients (22%) were at high-risk Rai stage (IV).

Comprehensive clinical information including treatment histories was available. Standard clinical criteria were used for the initiation of chemotherapy for all patients. The patients were followed up for a period ranging from 2-120 months.

Patients were subjected to:

1- Thorough history taking, full clinical examination, radiological examination including chest X ray, abdominal ultrasound and/or CT

scan were done whenever needed for proper clinical staging of the disease.

Patients were treated by one of the following lines of chemotherapy depending on age, performance status and stage of the disease:

- Chlorambucil (Clb) and prednisone: Clb was given orally at dose of 0.2 mg/kg/day and prednisone 20 mg/m^2/day.
- Cyclophosphmide, Vincristine, prednisone (CVP): Cyclophosphmide: 400 mg/m^2/IV on days 1-3, Vincristine: 1.4 mg/m^2 IV on day 1 and oral prednisone 100 mg/m^2 on days 1-5.
- Fludarabine and cyclophosphamide (FC): Fludarabine 25 mg/m^2 IV on days 1-3 and cyclophosphamide 250 mg/m^2 IV on days 1-3.

Response to induction chemotherapy was assessed according to the criteria proposed by the National Cancer Institute (NCI)-sponsored working group prior to study SWOG-9108 **[19]**.

Evaluation of response to chemotherapy had been made according to the following criteria:

Complete remission (CR):

Asymptomatic patients with no orgnomegaly or lymphdenopathy. Lymphocyte count < 4x 10^3/µl, neutrophils >1.5x10^3/µl, Hemoglobin (HB) >11 gm/dl, platelets count >100x10^6/µl and bone marrow lymphocytes < 30%.

Partial remission (PR):

More than 50% decrease in organomegaly or lymphadenopathy plus one of the following: neutrophils >1.5x10^3/µL, Hemoglobin >11 gm/dl and platelets >100x10^6/µl.

Progressive disease (PD):

New lesion or >50% increase in organomegaly or lymphadenopathy, circulating lympocytes revealing >50% increase.

Stable disease: Patients who do not fit the criteria for CR, PR, or PD **[19]**.

Time to disease progression (TDP): Duration of response was measured from the date of initial response until disease relapse or progression; or death from any cause, with observation censored at the date of last contact for patients last known to be alive without report of relapse **[21]**.

Overall survival (OS): Was measured from the date of presentation to the Medical Oncology Department until death from any cause, with observation censored at the date patients were last known to be alive for those not known to have died **[21]**.

2- Routine laboratory tests were also done including, liver and kidney function tests, uric acid and Coombs' test, LDH and $ß_2$ microglobulin ($ß_2$M).

3- Complete blood picture, bone marrow aspiration and biopsy.

4- Immunophenotyping using flowcytometer, Partec III to confirm the diagnosis of CLL with a wide panel of monoclonal antibodies including:

Fluorescein isothiocyanate (FITC) conjugated: CD45, CD3, CD4, CD20, FMC7, HLA-DR, and Kappa light chains.

Phycoerythin (PE) conjugated: CD5, CD23, CD10, CD22, CD56, CD79b, CD8 and lambda light chains.

Phycoerythin-Cyanine5 (PECya5) conjugated: CD19.

5- Immunophenotyping for the expression of our studied markers:

FITC conjugated: P53.

FITC conjugated: ZAP-70.

PE conjugated: CD38.

All monoclonal antibodies used were tested as surface expression except for, ZAP-70 and P53 in which we measured their cytoplasmic expression.

All monoclonal antibodies used were purchased from Dako except, ZAP-70 was (BD Bioscience). Results were expressed as a percentage of cells showing positive expression.

Intra-cytoplasmic staining of ZAP-70 and P53 proteins was done using the intrastain kit (purchased from Dako). As the antibody is directed against intracytoplsmic antigen, permeabilization of the cell membrane is necessary before incubation with antibody. Therefore, the kits contain two solutions: solution A is the (fixing) agent based on a paraformaldhyde solution and solution B is the (permeabilizing) agent based on a combination of lysing solution and detergent.

N.B the tube labeled for ZAP-70 contained ZAP-70/CD5/CD19, as anti-ZAP-70-FITC, anti-

CD5-PE and anti-CD19-PEcya5. This combination was done to quantify the cytoplasmic expression of ZAP-70 in B-CLL cells (CD5+, CD19+). As ZAP-70 is present in normal T (CD3+) and Natural killer (NK) cells (CD56+), so to exclude the ZAP-70 of normal T-lymphocytes (CD3+) and NK (CD56+) cells contamination. We can detect the level of ZAP-70 in CLL cells by gating on (CD19+, CD5+) B-CLL cells.

Interpretation of results:

For most studied markers, positive expression was considered when the marker is identified in more than or equal to 20%. ZAP-70 was defined as positive when identified in more than or equal to 20% of the gated CD19/CD5 positive cells **[14]** (Fig. 1). CD38 was considered positive when at least 30% of the gated (CD19+, CD5+) B cells expressed it **[18]**.

To establish the proper cut off value of P53% expression a Roc curve was done. Using this curve, a threshold of 4.95% was found to be appropriate for P53, above which the results were considered positive with a sensitivity of 60% and a specificity of 100%.

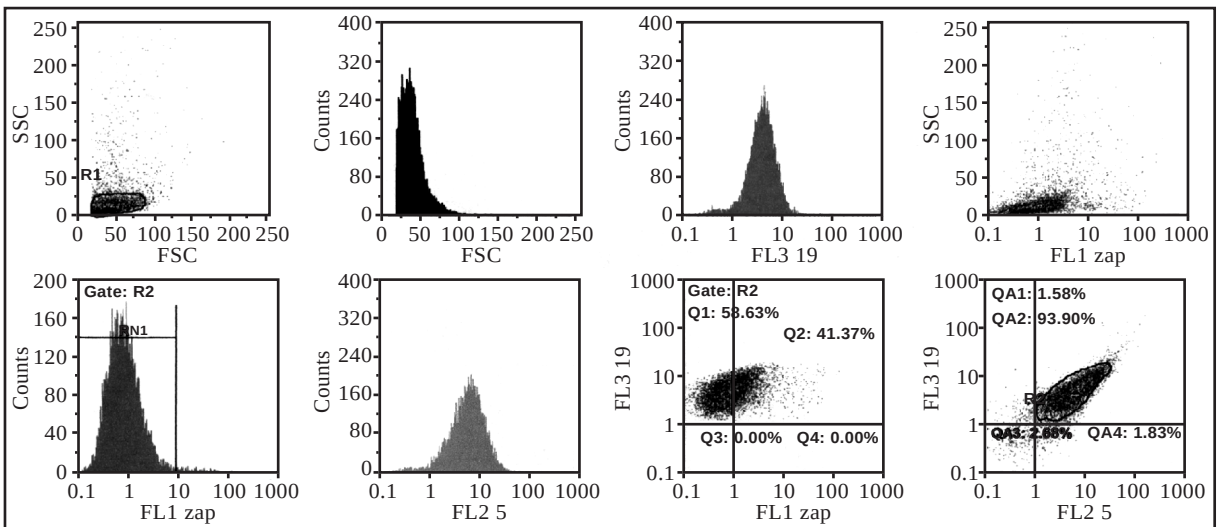
For CD38, results were also expressed as mean fluorescence index (MFI). This was done by dividing the mean fluorescence intensity of the test over that of the negative control **[22]**. Using ROC curve, a cut off value of 2.25 (with a sensitivity of 68% and a specificity of 70%) were found to be appropriate for CD38 MFI.

Statistical analysis:

Data was analyzed using SPSS win statistical package version 12. Numerical data were expressed as mean ± Standard deviation (SD), median, minimum and maximum. Qualitative data was expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables.

For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric one corresponding to the student *t*-test) for variables not normally distributed. While, comparison between 3 groups was done using one way ANOVA on rank of variables followed by Post-Hoc test (Scheffe test) for multiple comparisons.

Receiver Operating Characteristic (ROC) curve was plotted for determination of the cut off values of MFI-ZAP, MFI-CD38 variables. Survival analysis was done using Kaplan-Meier Method. Comparison between two survival curves was done using log-rank test. Probability (*p*-value) ≤ than 0.05 was considered significant and less than 0.001 considered as highly significant.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	(None)	8858	8858	–	91.68	39.51	41.46	31.93	7.41	8.71	59.84
Q1	R2	5300	4918	–	58.63	0.54	0.59	38.39	3.80	4.17	44.38
Q2	R2	3907	3470	–	41.37	2.07	3.37	231.15	5.99	6.71	47.05
Q3	R2	303	0	–	0.00	–	–	–	–	–	–
Q4	R2	152	0	–	0.00	–	–	–	–	–	–
R2	(None)	8388	8388	–	86.81	6.43	7.96	66.85	4.59	5.22	53.13
QA1	(None)	153	153	–	1.58	0.55	0.61	38.95	1.77	1.95	61.98

Fig. (1): Flowcytometric analysis of a case of CLL, ZAP-70 Positive.

RESULTS

The present study included 50 patients with chronic lymphocytic leukemia (CLL). They were 40 males (80%) and 10 females (20%) with a male to female ratio of 4/1. Their ages ranged from 37 to 80 years with a mean of 58.6±9.31 SD, (median = 57) years. In addition, 10 age and sex-matched normal volunteers were taken as a control group.

All patients were followed up for their initial response after 3-6 cycles of the initial chemotherapy and then have been followed up for a period of 2-120 months.

Clinical data of the studied group:

Rai staging system of CLL patients at the time of diagnosis showed, seven patients (14%) in stage I, 19 patients (38%) in stage II, 13 patients (26%) in stage III and 11 patients (22%) in stage IV. According to the modified Rai staging system, 26 patients (52%) were at low and intermediate risk (0, I, II), while 24 patients (48%) were at high risk (III, IV).

After the initial chemotherapy, 32/50 patients (64%) showed response to the initial treatment (CR 14 or PR 18), while 18/50 patients (36%) showed no response (SD 11 or PD 7).

During the follow-up period, 6/50 (12%) had died due to causes related to CLL while, 44/50 (48%) were alive at the end of the study.

Laboratory findings:

**ZAP-70 results:*

Our studied CLL group had a mean ZAP-70% expression of 20.98±18.3 SD. We found that 22/50 patients (44%) were positive while 28/50 patients (56%) were negative for ZAP-70.

Correlation of ZAP-70 expression to other known poor prognostic criteria of CLL (Table 1):

No significant relation was found when comparing ZAP-70% expression with age, sex or initial modified Rai staging at the time of diagnosis (p=0.802, 0669 and 0.164 respectively).

On the other hand there was a significant negative relation of ZAP-70% expression with initial response to chemotherapy (p<0.001).

A significant positive relation was also found between ZAP-70% expression and the diffuse infiltration pattern of bone marrow biopsy p=0.002.

A significant increase in serum levels of LDH and $ß_2$M in ZAP-70 positive groups as compared to ZAP-70 negative groups was detected (p=0.049 and 0.007 respectively). There were non significant differences in TLC, HB and PLT values between ZAP-70 positive and ZAP-70 negative groups (p=0.356, 0.325 and 0.599 respectively).

A significant positive relation was found between ZAP-70% expression and P53% expression (p=0.005).

There was no significant relation between ZAP-70% expression and Bcl2% expression, (p=0.641).

Regarding survival status, six CLL related deaths occurred during the observation period and 5 patients of this group were ZAP-70 positive (83.3%). In contrast to only 1/6 in the ZAP-70 negative group (16.7%). In the ZAP-70 positive group 5/22 (22.7%) patients had died in contrast, only 1/28 (3.5%) patient in the ZAP-70 negative group.

The relation between the two groups showed statistically near significance (p=0.075).

** CD38 results:*

The mean CD38% expression of the studied group was 13.2%±14.3 SD (positive cases were 9/50 cases). The mean CD38 MFI expression of the studied group was 12.4±11 SD (positive cases were 22/50 cases).

Correlation of CD38% expression and the other bad prognostic criteria of CLL (Table 1):

There was no significant relation between CD38 expression on one hand and either age, sex, bone marrow infiltration pattern or modified Rai stage at diagnosis on the other hand (p= 1.00, 0.09, 0.428 and 0.721 respectively).

A significant negative relation was found between CD38% expression and the initial response to chemotherapy (p=0.034).

When measuring CD38 as percentage of cell expression, a significant increase in serum levels of B_2M in CD38 positive group as compared

to CD38 negative group was encountered (p= 0.045). A non significant differences was found for TLC, HB, PLT, LDH, P53 and Bcl2 in CD38 positive as compared to CD38 negative groups (p=0.960, 0.419, 0.733, 0.105, 0.802 and 0.305 respectively).

No significant relation was found between CD38% expression and ZAP-70% or p53% expression (p=0.441, 0.802 respectively).

Correlation of CD38 MFI to the other poor prognostic markers in CLL patient group: A significant positive relation was found with advanced modified Rai staging at diagnosis and with disease progression (DP) (p=0.019 and 0.033 respectively) and a negative significant relation with good initial response to chemotherapy (p=0.006).

A significant increase in TLC, LDH and $ß_2$M levels was found in CD38 MFI positive group as compared to CD38 MFI negative groups (p=0.020, 0.033 and 0.05 respectively). There was a significant decrease in HB level between CD38 MFI positive and negative groups (p=0.015). No significant differences was found in PLT, P53% and Bcl2% expressions in CD38 MFI between the two groups (p=0.178, 0.358 and 0.679 respectively) (Table 1).

Table (1): Correlation of ZAP-70%, CD38% and CD38 MFI with clinical and laboratory data in the studied 50 B-CLL cases.

	ZAP-70% Cut off 20% Positive cases: 22/50	CD38% Cut off 30% Positive cases: 9/50	CD38 MFI Cut off 2.25 Positive cases: 22/50
Rai staging at diagnosis:			
• 0, I, II	N.S.	N.S.	p=0.019
• III, IV			
Initial response to chemotherapy:			
• CR, PR	p<0.001	p=0.034	p=0.006
• SD, PD			
Bone marrow infiltration pattern:			
• Diffuse	p=0.002	N.S.	N.S.
• *Non-diffuse:*			
TLC	N.S.	N.S.	p=0.02
Hb	N.S.	N.S.	p=0.015
Platelet count:	N.S.	N.S.	N.S.
LDH	p=0.049	N.S.	p=0.03
B_2M	p=0.007	p=0.045	p=0.05
P53%	p=0.005	N.S.	N.S.

N.S.: Non significant. p value: Significant ≤0.05.

Results of the combined analysis of (ZAP-70 and CD38):

Our CLL patients were classified according to the combined expression of ZAP-70% cut off 20% and CD38% cut off 30% into 3 main groups:

The first group with concordant ZAP-70+, CD38+), which included 5/50 patients (10%).

The second group with discordant (ZAP-70+, CD38-) and (ZAP-70-, CD38+), which included 21/50 patients (42%).

The third group with concordant (ZAP-70–, CD38-), which included 24/50 patients (48%).

Table (2): Comparison of clinical and laboratory data between ZAP-70/CD38 concordant and discordant group in the studied 50 B-CLL patients.

Parameter	ZAP-70 and CD38 Total (n) 50			p value
	Concordant both positive	Discordant one positive, one negative	Concordant Both negative	
Number of patients	5 (10%)	21 (42%)	24 (48%)	
Rai stage diagnosis:	a	a	b	
(0.I, II)%	2/5 (40%)	9/21 (42.8%)	15/24 (62.5%)	0. 396
(III, IV)%	3/5 (60%)	12/21 (57.1%)	9/24 (37.5%)	
Initial response:	a	b	c	
(CR, PR)%	0/5 (0%)	11/21 (52%)	21/24 (87.5%)	<0.001
(SD, PD)%	5/5 (100%)	10/21 (48%)	3/24 (12.5%)	
Bone marrow biopsy:	a	b	c	
Diffuse%	4/5 (80%)	12/21 (57.1)	5/22 (22.7%)	0.015
Non-diffuse%	1/5 (20%)	9/21 (42.8%)	17/22 (77.2%)	
LDH (IU/L):	a	b	c	
Mean (n)	914 (n=4)	716 (n=19)	543 (n=20)	0.029
B_2M (mg/L):	a	a	b	
Mean (n)	3.7 (n=4)	3.5 (n=18)	2.2 (n=16)	<0.001
P53%:	a	b	c	
Mean (n)	25 (n=3)	10 (n=13)	3 (n=15)	0.050

*The p-value for comparison among the three subgroups, was calculated using the one way ANOVA on rank of variables followed by Post-Hoc test.

The concordant both positive group showed decreased response to chemotherapy, more diffuse pattern of bone marrow infiltration and increased

LDH, B_2M levels and P53%. The concordant both negative group showed increased response to chemotherapy, non diffuse pattern of bone marrow infiltration and lower LDH, B_2M levels and P53%. The discordant group showed intermediate response to chemotherapy, less diffuse pattern of bone marrow biopsy than the first group and LDH, B_2M and P53% levels intermediate between the other two groups.

Relation of time to disease progression (TDP) with different parameters in the study:

In our studied CLL patients 32/50 responded to the initial chemotherapy while, 18/50 patients showed no response. The follow-up period of TDP of CLL patients ranged between 2-120 months.

The cumulative survival (CS) at 1 year was 79% (for ZAP-70 negative patients, compared to 42% in ZAP-70 positive patients (p=0.020) (Fig. 2).

In CD38 negative patients with CS at one year was 88%, which was significantly higher when compared to CD38 positive patients, the CS at 1 year was 33% (p=0.033) (Fig. 3).

No patients in the concordant both positive markers responded to the initial chemotherapy. In concordant both (ZAP-70 and CD38) negative group, the CS was 86%, the median was 29 months. In the discordant (ZAP-70+ and CD38– or ZAP-70– and CD38+) group the CS was 41%, the median was only 12 months. There was a significant relation between the combined CD38 and ZAP-70% expression groups and TDP (p<0.001) (Fig. 4).

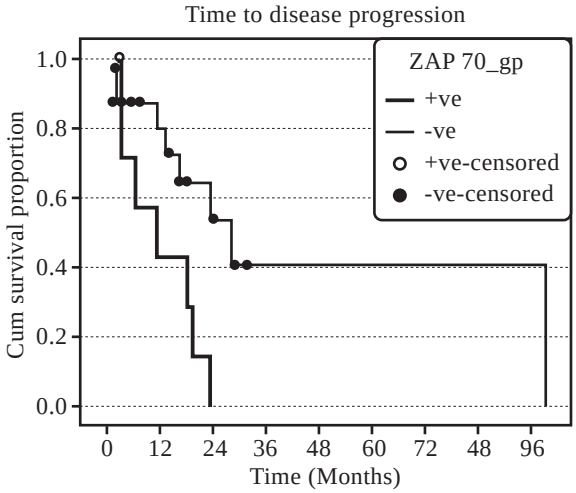


Fig. (2): Time to disease progression in relation to ZAP-70 groups (p=0.020).

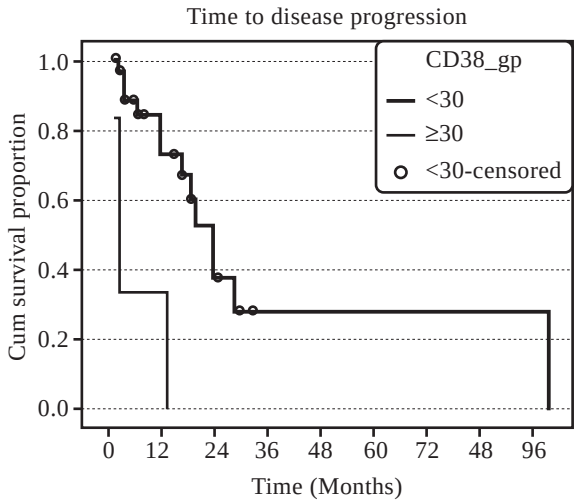


Fig. (3): Time to disease progression in relation to CD38 groups (p=0.033).

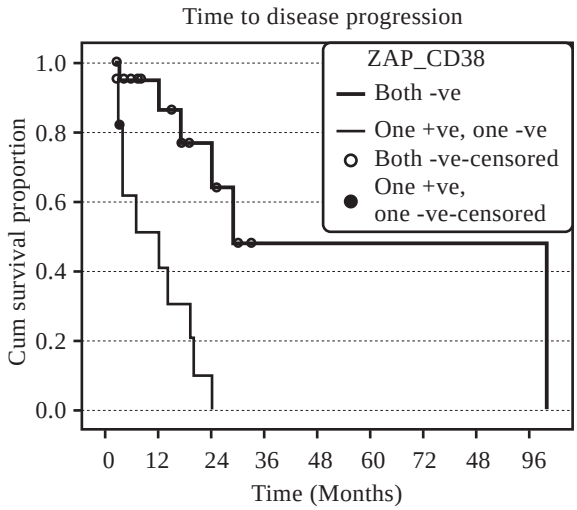


Fig. (4): Time to disease progression in relation to ZAP-70/CD38 groups (p<0.001).

Survival study:

The follow-up period of CLL patients ranged between (2-120) months with a median follow-up of 18 months. The cumulative overall survival (CS) was 89% with a mean of 95.3±11.75 SD, with (95% confidence interval (CI) 72-118%). The median survival was not reached as >50% of cases (88%) was still alive at the end of the study.

Survival analysis regarding the expression of the studied markers was as follows:

The cumulative overall survival (CS) at 1.5 year was 96% for ZAP-70 negative patients, but only 81% for ZAP-70 positive patients. This difference was statistically significant (p=0.029) (Fig. 5).

At 1.5 years the CD38+ CLL patients had a cumulative overall survival (CS) 76% compared to CD38 -ve patients with CS 92%. However, this difference was not statically significant (p=0.1970) (Fig. 6).

In concordant both ZAP-70 and CD38 positive group, the CS was 80%. In the discordant (ZAP-70+ and CD38– or ZAP-70– and CD38+) group the CS was 81%. In the concordant both ZAP-70 and CD38 negative group the cumulative survival was 100% since all patients in this group were alive at the end of the study. This relations between the combined (CD38 and ZAP-70) expression groups and overall survival were statistically significant (p=0.031) (Fig. 7).

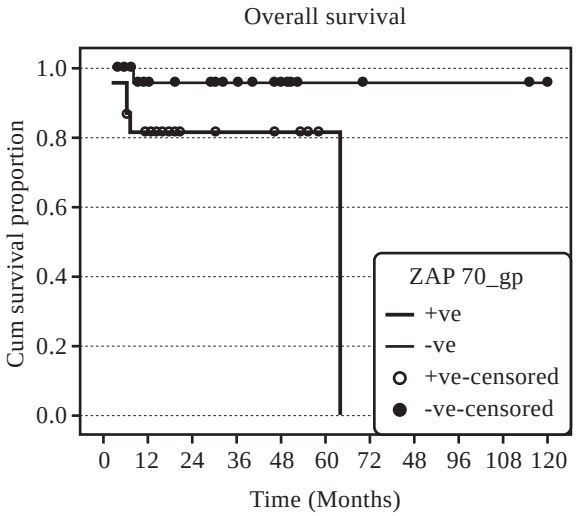


Fig. (5): Overall survival in relation to ZAP-70 percentage expression (p=0.029).

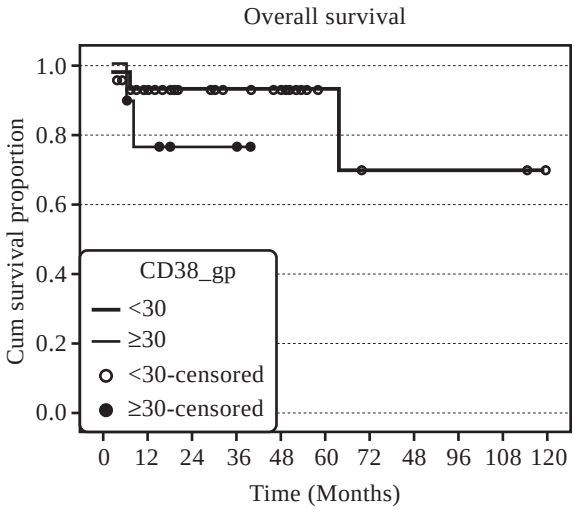


Fig. (6): Overall Survival in relation to CD38 percentage expression (p=0.1970).

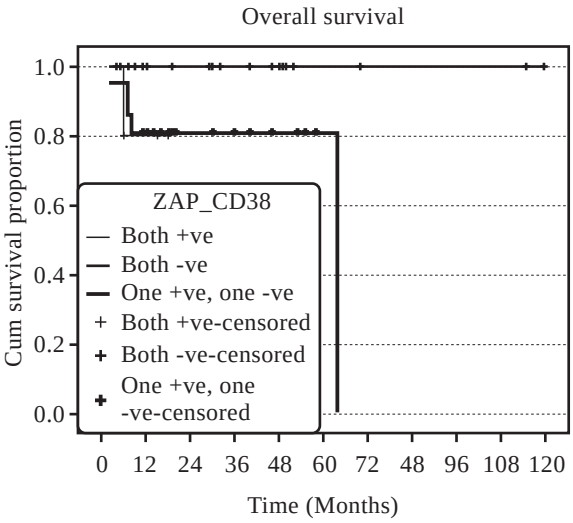


Fig. (7): Overall survival in relation to ZAP-70/CD38 groups (p=0.031).

DISCUSSION

Chronic lymphocytic leukemia (CLL) is defined as a proliferation of B lymphocytes that express surface CD19 or CD20, CD5, CD23, and low levels of immunoglobulin (Ig), CD79b, and CD22 **[23]**.

In Egyptian National Cancer Institute (NCI), CLL constituted 0.4% out of 556 cancer cases and 7.4% out of 621 leukemic cases registered during the year of 2001 **[24]**. In 2005, the male CLL/leukemia was 20/311 (6.4%) and constituted 0.5% out of all cancer cases. In female CLL/leukemia was 26/414 (6.3%) and constituted 0.4% out of all cancer cases **[25]**.

Heterogeneity in the clinical behavior of CLL makes it difficult to identify, which patients will benefit most from earlier or more aggressive treatment and those who should be treated with more conservative and less toxic approaches. Clinical researchers have long thought to identify a marker (or markers) for use as a prognostic tool **[3]**.

ZAP-70, a member of the syk family of tyrosine kinases, has demonstrated an equivalent clinical utility to IgH mutational status in correlation with disease progression and survival **[26]**.

CD38 has been associated with disease progression in many different types of leukaemias, including B-CLL **[22]**.

The present study included 50 patients with chronic lymphocytic leukemia (CLL). They were 40 males (80%) and 10 females (20%) with a male to female ratio of 4/1. Their ages ranged from 37 to 80 years. In addition, 10 age and sex-matched normal volunteers were taken as a control group.

ZAP-70 expression:

In this study, when using the percentage expression for interpretation, the leukemic cells were ZAP-70 positive in 22/50 (44%) and ZAP-70 negative in 28/50 (56%). These results were similar to recently reported researches **[27,28]**. However, a higher percentage of positivity was detected when using MFI, suggesting a higher sensitivity for this method.

A significant differences were found between ZAP-70 positive and ZAP-70 negative patients with some of B-CLL poor prognostic parameters (LDH and B_2M p=0.049 and 0.007 respectively). This is in accordance with what was reported earlier **[27,29,30]**.

Our results showed a positive significant relation between ZAP-70% expression and the diffuse pattern of bone marrow biopsy (p= 0.002). This is in agreement with recent studies which reported that the expression of ZAP-70 was related to the infiltration type **[27,28]**.

A significant negative relation was found between ZAP-70% and the initial response to chemotherapy (p<0.001). These results are in accordance with what was previously reported **[1,32,33,34]**. In addition, our results are in agreement with the earlier results, which found that ZAP-70 positive patients required more intensive chemotherapy over longer periods of time than ZAP-70 negative patients **[14]**.

This study showed a positive relation between ZAP-70 and P53% expression (p=0.005) with no significant relation with Bcl2 and this relation was in accordance with previous studies **[31,33,35]**.

Six CLL-related deaths had occurred during the observation period. Five out of those six patients were ZAP-70+ (83.6%). In the ZAP-70 positive group 5/22 (22.7%) patients had died during the follow up period In contrast only 1/28 (3.5%) patients was in the ZAP-70 negative group. The difference between the two groups was statistically of near significance (p= 0.075). Our results are in agreement with that of other researchers, who reported that, 15/156 CLL-related deaths occurred during their observation period and 12/15 patients of this group were ZAP-70+ (80%). In contrast only 3/15 patients deceased in the ZAP-70– group (20%) **[30]**.

ZAP-70 was proven by many authors to be a sensitive and specific surrogate marker for IgV_H mutational status though, the mechanisms accounting for the relation between both factors remain unknown **[26,36]**. Moreover, the prognostic value of ZAP-70 expression is even more significant than IgV_H mutational status **[13]**.

CD38% expression:

In B-CLL cells, CD38 was considered of prognostic value when ≥30% of (CD19+, CD5+) B CLL cells expressed this membrane antigen **[37]**. Based on the current study using this cut off value, 9/50 (15%) were positive while 41/50 (85%) were negative. The low percentage of CD38 positivity may be attributed to the fact that most of our patients (80%) had received many cycles of chemotherapy before the time of testing. Previous studies have reported that CD38, expression can change with time and under different conditions and chemotherapy selectively eliminate the CD38 clone **[18]**. It was therefore suggested for the accurate assessment of the prognostic significance of CD38 positivity is to ensure that only samples close to or at the time of presentation are tested **[22]**.

Our results were more or less similar to previously reported ratios **[29,38]**. This can be explained as most of their patients had received chemotherapy at the time of analysis. In contrast, a different ratio was obtained in a more recent study, where CD38 positivity was observed in 89% of the studied CLL cases. However, none of his patients had received chemotherapy at the time of sampling **[27]**.

In this study, no significant relation was found between CD38% expression and age, sex, the infiltration pattern of bone marrow biopsy or modified Rai staging at diagnosis. Also, no significant relation was found between CD38% expression and P53% or Bcl2% expressions and these results are in accordance with other studies **[22]**.

A significant negative relation was found between CD38% expression and the initial response to chemotherapy (p=0.034). Only 3/9 (33.3%) of CD38% positive cases responded to chemotherapy either by CR or PR; in contrast 6/9 (66.7%) did not respond either by SD or PD. These results are in agreement with recent studies **[30,33,38]**.

Our results showed a significant increase in serum level of $ß_2$M in CD38 positive as compared to CD38 negative (p=0.045). These results were in accordance with previous results **[22,29]**.

In the present study, no significant differences were found between CD38% positive and CD38% negative cases regarding TLC. This is in accordance with what was reported earlier **[39]**.

By using the mean fluorescent intensity for measuring CD38, a positive significant relation was found between advanced modified Rai Staging at diagnosis and CD38 MFI (p=0.019).

A negative significant relation was found between initial response to chemotherapy and CD38 MFI (p=0.006).

No significant relation was found between CD38 MFI and the other bad prognostic markers and this was in accordance with previous studies **[22]**.

On comparing CD38 MFI positive and negative cases of the studied CLL patients, a significant difference was found regarding TLC, HB, LDH and $ß_2$M. Patients with CD38 MFI positive cases showed increased TLC, decreased HB, increased LDH and B_2M (p=0.02, 0.015, 0.033 and 0.05 respectively) as compared to CD38 MFI negative cases.

As a result, we can conclude that, semiquantification of the CD38 antigen by flowcytometry greatly improves the prognostic value of the percentage expression and this is in accordance with previous reports **[22,40]**.

Combined ZAP-70 and CD38 analysis:

Combined analysis of (ZAP-70 and CD38) allowed separation of our patient cohort into 3 subgroups, that were, concordant ZAP-70–, CD38– (42%), concordant ZAP-70+ CD38+ (10%) and patients with discordant results either (ZAP-70+, CD38– or ZAP-70–, CD38+) (48%). Importantly, these three subgroups differed in their clinical characteristics and laboratory findings. The combination of ZAP-70 and CD38 increased the prognostic power of both factors.

A significant relation was found between the three subgroups with the initial response to chemotherapy (p<0.001), pattern of bone marrow biopsy (p=0.015), serum level of LDH (p= 0.029) and $ß_2$M (p<0.001) and P53% expression (p=0.05).

There was no relation between the three subgroups and the advanced modified Rai staging at the time of diagnosis.

No significant relation was found between the three subgroups and sex or age. All these results are in accordance with other reports **[14, 29,30,41]**.

A negative significant relation was found between the OS and the combined expression of the both markers (p=0.031). The worse OS was found in the concordant both positive groups with CS 80%. Intermediate survival was found in the discordant both markers with CS 81% and the best survival was found in the concordant both negative groups with CS 100%. These results are in accordance with previous researches **[14,27,29,30]**.

Also a negative significant relation was found between TDP and the combined expression of both markers (p<0.001). The longest TDP was found in the concordant both negative group (median 29 months and CS 86%), intermediate TDP was found in the discordant both markers (median 12 months and CS 41%) and the shortest TDP was found in the concordant both positive group. These results are in agreement with recent studies **[27,30]**.

Conclusion:

ZAP-70 positive B-CLL group has a decreased response rate, a worse stage of the disease, a decrease in overall survival and less TDP than that of ZAP-70 negative patients. This suggests that ZAP-70 assay is a powerful predictor of outcome in CLL.

On comparing the two methods used for evaluating CD38 expression, (percentage expression and MFI), it revealed that the use of both MFI in association with percentage positivity can be a better predictor of disease pro-

gression and outcome of the disease than the percentage alone.

Our data showed that the prognostic information given by ZAP-70 and CD38 expression is complementary. Combined analysis of these two markers allows for the separation of three B-CLL patient subgroups with good, intermediate and poor prognosis and therefore could be used to guide treatment decisions especially in early clinical stages of the disease. Since flowcytometry can be used reliably to assess CLL samples for ZAP-70 and CD38, it should be more suitable for application in clinical laboratories than IgV_H mutation analysis, which is a technically more demanding and expensive assay to perform.

Recommendations:

The expression of ZAP-70 is a good prognostic marker and should be used in the routine work up of CLL.

CD38 can change with time and under different conditions, as chemotherapy selectively eliminates the CD38 clone. It is important therefore, for the accurate assessment of the prognostic significance of CD38 positivity to ensure that only samples close to or at the time of presentation are tested before taking any type of chemotherapy.

We recommend the adoption of MFI. Wherever, CD38 positivity is currently measured as part of the clinical assessment of CLL patients. Trials should be performed in different centers to evaluate the prognostic significance of quantification of CD38.

However, further studies are required to develop a standardized flowcytometry protocol that will allow comparison of ZAP-70 and CD38 measurements between different laboratories.

Finally, the prognostic value of combined ZAP-70/CD38 analysis should be tested in the setting of a controlled prospective trial. Performing this combined analysis could serve for either precise definition of prognostic subgroups or might be an option to confirm the prognosis if the value of one predictive factor is borderline.

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