ZAP70 and CD38 as Prognostic Markers for Chronic Lymphocytic Leukemia in Egyptian Patients

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Abstract

Background: Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in elderly people, characterized by progressive lymphocytosis caused by the clonal accumulation of CD5, CD19 & CD23 B cells in peripheral blood, bone marrow and lymphoid organs. The most important prognostic factors in CLL are clinical stage, markers of tumour load (e.g., thymidine kinase (TK), β_2-microglobulin (β_2MG) and cellular proteins expression (eg. CD3 8 and ZAP70).

Aim of Work: To identify prognostic markers (CD3 8 and ZAP-70) in Egyptian CLL patients in order to investigate the impact of their combined expressions on the treatment outcome and quality of life.

Material and Methods: Thirty CLL patients and 20 healthy controls were enrolled in the study. Blood samples of patients and controls were analyzed for the expression of surface CD38 and cytoplasmic ZAP-70 by flowcytometry.

Results: 18 patients (60%) out of 30 showed high level of ZAP-70 and CD38 above the cut off value, while the remaining (40%) showed lower level of ZAP-70 and CD38.

Conclusion: There was a statistical significant difference between stages in the two groups (ZAP-70 +/CD38+ and ZAP-70$^-$/CD38$^-$). Positive cases showed higher prevalence of Stages III and IV while negative cases showed higher prevalence of Stage II with p-value (<0. 00 1 *). As regards survival analysis, there was no statistical significant difference between the two groups, however; positive cases showed higher median and mean survival period than negative cases with p-value (0.504).

Key Words: *CLL – ZAP- 70 – CD38 – Flowcytometry.*

Introduction

CHRONIC Lymphocytic Leukemia (CLL) is the most common leukemia in adults which comprises approximately 16-30% of all leukemia cases [1]. It is more common in males and mostly occurs after the age of 60 years [2]. Disease progress is quiet variable according to a number of factors [3].

The Rai and the Binet systems are two commonly accepted staging methods in CLL [4,5]. Although Rai staging system predicts overall survival, it does not predict which patients in the early stages (0-I) will progress and require therapy [6]. The traditional prognostic parameters based on routine and well established tests involving the blood or bone marrow (clinical stage, pattern of bone marrow infiltration, lymphocyte doubling time, beta-2 microglobulin levels, and lactate dehydrogenase level) are useful but they may not accurately predict progression for a given patient [7].

The clinical behavior of patients with CLL is heterogeneous. Some patients have indolent disease without complications for many years; others develop progressive disease requiring therapy within a short time after diagnosis. Early treatment of the former could lead to therapy related complications that might compromise their quality of life and/or survival [8]. Defining markers that reliably can stratify patients into groups with good-risk or poor-risk disease could facilitate clinical trials evaluating the potential benefit of early treatment [9].

Recently focus of research on prognostic factors in CLL has changed from clinical to biological factors. The immunoglobulin Variable Heavy Chain (IgVH) mutational status, CD38 and ZAP-70 expression are rarely used as markers [10].

ZAP-70 is a member of the syk family Protein Tyrosine Kinases (PTKs) which plays a critical role in T-cell antigen receptor (TCR) signaling and T-cell development [11]. It was found also to be associated with the B-cell receptor (BCR) in CLL.

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CLL cells expressing high levels of ZAP-70 were found to be associated with enhanced signal transduction via the BCR complex, when compared to those with low expression of ZAP-70 [12].

CD38 is a 45-kDa, nonlineage-restricted, type II transmembrane glycoprotein that has many protein functions. It can serve as an ectoenzyme that catalyzes the synthesis and hydrolysis of cyclic ADP-ribose, a $Ca2+$ mobilizing agent that acts independently of inositol triphosphate [13]. CD38 also functions as a receptor that induces proliferation and increases survival of CLL cells [14]. CD38 positivity (defined as at least 30% positive cells) is an independent prognostic marker for an unfavorable clinical course in CLL [15]. CD38 positive patients may progress faster to advanced stage. These patients not only have more aggressive disease but also do not respond to chemotherapy as others do [16].

Material and Methods

Informed consent was obtained from all participants. Ethical approval was obtained by the Institutional Review Board (IRB) of Clinical Oncology Department; Cairo University. Thirty newly diagnosed patients with CLL were included in the study, in the period from September 2012 to July 2014. Patients were diagnosed and treated in the Clinical Oncology Department, Kasr AL-Aeny, School of Medicine Cairo University. Twenty healthy controls were also enrolled.

Patients were subjected to:

- Thorough history taking, full clinical examination and radiological investigations.

- Routine laboratory tests including complete blood picture (CBC), Liver Function Test (LFT), Kidney Function Test (KFT) as well as uric acid were done.

- Immunophenotyping using flowcytometery to confirm the diagnosis of CLL with a wide panel of monoclonal antibodies (CD5, CD19, CD23, CD79b and FMC7) as well as expression of understudy markers (ZAP-70 and CD38) as prognostic markers. All monoclonal antibodies used were tested as surface expression except for ZAP-70 that was tested for cytoplasmic expression with an intrastain kit provided with a permeabilization agent.

Flow cytometric detection of CD38:

Flow cytometric analysis of CD38 was performed on fresh PB samples stained with CD5-Fluorescein Isothiocyanate (FITC), CD19-ECD, and CD38-Phycoerythrine (PE). Isotype controls were run with each sample to distinguish positive from negative cells. CLL cells (CD5 +CD 19+) were gated, and CD38+ cells were measured in CD5 + CD19+ lymphocyte population. A FACS calibur flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences) were used to acquire and analyse data. The cut-off point for CD38-positive in CLL cells was >30%.

Flowcytometric detection of ZAP- 70:

Cytoplasmic ZAP-70 expression was determined by flowcytometry on fresh PB samples. Cells were fixed and permeabilized using the Fix and Perm kit (Caltag) according to the manufacturer's instructions. CD5-FITC (BD Biosciences) and CD19 ECD (BD Biosciences) were used for staining samples. Lymphocyte cells were gated further to select CD5 + CD 19− cells (T cells), which were used as an internal positive control, and CD5+CD19+cells (CLL cells). Isotype controls were run with each sample to distinguish positive from negative cells. Data acquisition and analysis were performed using a FACS Calibur flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences). After appropriate lymphocyte gating, a marker that included >97% of the T cells (positive control) was used to define the percentage of CLL cells that expressed ZAP-70 with the same intensity of fluorescence as the T cells. The cut-off point for ZAP-70 positivity in CLL cells was >20%.

- *Statistical analysis of data collected:*

Quantitative data were presented as Mean (M), median, Standard Deviation (SD) and range values. Age and Hemoglobin (Hb) level data showed parametric distribution; Student's *t*-test was used for comparison between the two groups regarding these variables. All other data showed non-parametric distribution; Mann-Whitney U test was used for comparisons between the two groups.

Qualitative data were presented as frequencies and percentages. Chi-square (X^2) test was used for comparisons between the two groups.

Spearman's correlation coefficient was used to determine significant correlations between immunophenotyping findings and blood picture findings.

Kaplan-Meier survival curve was constructed for survival analysis to estimate the mean survival time.

The significance level was set at $p < 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

Overall survival was examined in our study, it is the time measured from the diagnosis date to either the last follow-up date or the time of death by any cause.

Results

Patients characteristics:

Patients group showed a statistically significant higher mean of TLC, platelets count than control group but showed lower mean of Hb than control group (Table 1).

Immunophenotyping:

Patients group showed a statistically significant higher mean level of ZAP70 and CD38 than control group (Table 2).

Correlation between ZAP70 and different Peripheral blood parameters:

A statistically significant correlation was found between ZAP70 and different PB parameters except for stage of disease where there was a statistically significant positive (direct) correlation between the stage of disease and ZAP70 (Table 3).

Correlation between CD38 and different peripheral blood parameters:

There was a statistically significant negative (inverse) correlation was found between CD38 and age as well as Hb level while a significant positive (direct) correlation between CD38 and stage, with no statistically significant correlation between CD38 and other variables (Table 4).

Correlation between CD23 and different variables:

There was no statistically significant correlation between CD23 and different variables: Age, Hb-platelets and lymphocytes count) except for TLC where there was a statistically significant positive (direct) correlation between CD23 and TLC Fig. (1).

By comparing the hematological data studied, staging and survival in CLL patients with positive or negative ZAP-70 and CD38, we found that, there was no statistically significant difference between TLC, Hb, platelets and lymphocytes count in both cases. However, ZAP-70, CD38 positive cases showed higher of Stages III and IV while negative cases showed higher prevalence of Stage II and this response was statistically significant (Table 5). As regards survival time analysis, there was no statistically significant difference between the two groups, however; positive cases showed higher median and mean survival times than negative cases [(Table 5) and Fig. (2)].

Table (1): Clinical characteristics of CLL patients and control subjects.

Characteristics	Patients (n=30)	Control (n=15)	*p*-value
Hb (g/dl):			
M ± SD	10.0±1.5	11.9±1.0	<0.001*
Median	9.9	11.9	
Range	6.4-12.8	10.0-13.2	
TLC (X 10 ^{9}/L):			
M ± SD	119933.3±126069.2	5885.7±1048.0	<0.001*
Median	57650.0	5800.0	
Range	1200.0-470000.0	4300.0-7800.0	
PLT count (X 10 ^{9}/L):			
M ± SD	157703.1±110758.0	241.4±43.1	<0.001*
Median	131500.0	242.5	
Range	94.0-604000.0	169.0-310.0	

M : Mean.
Hb : Hemoglobin.
TLC : Total leukocyte count.
PLT : Platelets.
SD : Standard deviation.
*   : $p<0.05$.

Table (2): Comparison between immunophenotyping in patient and control groups.

Immuno-phenotyping	Patients (n=30)	Control (n=15)	*p*-value
ZAP 70 (%):			
M ± SD	34.3±29.1	8.6±4.0	0.020*
Median	33.6	8.6	
Range	1.0-95.1	1.4-15	
CD38 (%):			
M ± SD	35.2±26.1	17.2±25.6	0.024*
Median	34.0	10.9	
Range	0.3-95.7	3.2-105.0	

SD: Standard deviation.
* : $p<0.05$.
M : Mean.

Table (3): Correlation between ZAP-70 and different peripheral blood parameters.

PB parameters	Correlation coefficient	*p*-value
Age	–0.059	0.758
Hb (g/dl)	–0.273	0.144
TLC (X 10 9/L)	–0.159	0.400
PLT (X 10 9/L)	0.097	0.609
Lymphocytes %	–0.028	0.883
Stage	0.711	<0.001*

Hb   : Hemoglobin.
TLC : Total leukocyte count.
PLT : Platelets.
*   : $p<0.05$.

Table (4): Correlation between CD3 8 and different peripheral blood parameters.

PB parameters	Correlation coefficient	*p*-value
Age	–0.426	0.019*
Hb (g/dl)	–0.363	0.048*
TLC (X 10 9/L)	–0.223	0.235
PLT (X 10 9/L)	0.039	0.837
Lymphocytes %	–0.280	0.134
Stage	0.704	<0.001*

Hb   : Hemoglobin.
TLC : Total leukocyte count.
PLT : Platelets.
*   : $p<0.05$.

Table (5): Comparison between patients groups in relation to ZAP-70 & CD38 with hematological parameters and disease stage.

	ZAP-70+ & CD38+	ZAP-70⁻ & CD38⁻	p-value
TLC (X1 0 ^{9}/L):			
Mean ± SD	121823.5±145219.0	129790.9±105970.7	0.707
Median	45000.0	93100.0	
Range	6500.0-470000.0	1200.0-301000.0	
Hb (g/dl):			
Mean ± SD	9.8±1.5	10.6±1.5	0.164
Median	9.9	10.8	
Range	6.4-12.8	8.7-12.7	
PLT (X1 0 ^{9}/L):			
Mean ± SD	161882.4±96869.0	146000.0±65685.6	0.707
Median	132000.0	119000.0	
Range	52000.0-404000.0	52000.0-277000.0	
Lymphocyte (%):			
Mean ± SD	79.1±13.8	81.7±8.4	0.579
Median	83.0	85.0	
Range	50.0-98.0	60.0-98.0	
Staging (n, %):			
Stage II	1 (5.9)	9 (81.8)	<0.001*
Stage III	11 (64.7)	1 (9.1)	
Stage IV	5 (29.4)	1 (9.1)	
Survival:			
Median	47.0	34.0	0.504
Mean	52.0	40.2	

Hb   : Hemoglobin.   N: Number.
TLC : Total lymphocyte count.   * : Significant at $p \le 0.05$.
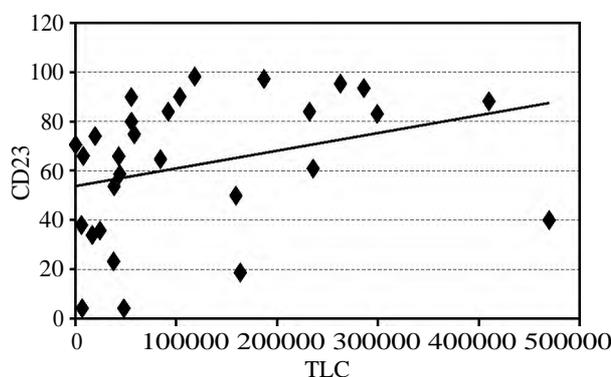PLT : Platelets.

Fig. (1): Scatter diagram showing positive correlation between CD23 and TLC.
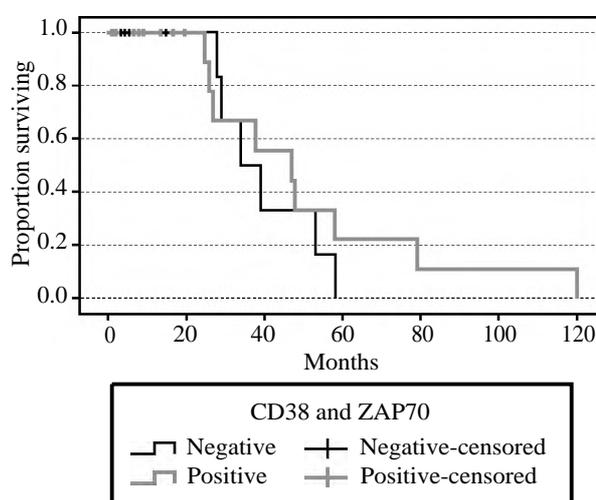
Fig. (2): Kaplan-Meier survival time curve for positive and negative CD38 and ZAP-70 cases.

Discussion

In CLL the current challenge is the identification of CLL patients at high risk, at time of diagnosis, for therapy-tailoring [17] CD38, a marker associated with CLL, was found to correlate with IgHV mutational status [18]. Recently, ZAP-70 was shown to be highly expressed in CLL patients with unmutated status and to be correlated with disease progression, and overall survival [19]. Thus assays for CD38 and ZAP-70 expression would yield important prognostic information for patients with CLL [20].

The present study included 30 CLL patients their age ranging from 35 to 79 years with median age 56 years. In addition to 20 controls ranging from 20-25. Patients PB samples were subjected to immunophenotypic analysis by flowcytometry for both diagnostic and prognostic markers namely ZAP-70 and CD 38.

In our study regarding ZAP-70 expression we found that there was no significant correlation between ZAP-70 and age, Hb, TLC and lympho-cytes count which is in agreement with [10]. Who reported that there was no correlation of ZAP-70 with (age, Hb and lymphocyte count). While in disagreement with [20]. Who reported that ZAP-70 levels were significantly positively correlated to TLC and absolute lymphocytic count in PB while it was negatively correlated with Hb levels and PLT.

Also, our study showed that there was a significant correlation between ZAP-70 expression and stage of disease which is in agreement with the previously reported data of [15,21], but in disagreement with [3,10] who reported that there was no correlation between ZAP-70 and stage of disease.

When we classified our patients into 2 groups according to their ZAP-70 status, we found that there was no significant difference between (TLC, Hb, PLT count and lymphocytes count) with positive or negative ZAP-70 which is in disagreement with [22] who reported that ZAP-70+ patients had significantly higher (TLC and lymphocyte count).

In addition, there was also no significant correlation between CD38 expression and the blood parameters. This is in disagreement with [22]. Who reported that there was significant positive correlation between CD38 expressions and (TLC count and lymphocytes count).

Also, the study revealed that there was a statistically significant negative correlation between CD38 and (Hb and age), these results are in agreement with the previously reported data from [20] who reported that CD38 levels were correlated negatively to Hb levels. While these data are in disagreement with [23] who reported that no significant correlation was found between CD38 expression and age and with [20] who found that no significant correlation between CD38 expression with age.

Our study showed also that there was significant positive correlation between CD38 expression and stage of disease ($p < 0.001$) this is in accordance with what was reported earlier by [22] while in disagreement with [23] who reported that there was no significant correlation between CD38 expression and modified Rai stage at diagnosis.

The present study revealed that advanced Rai stages (stage 3 and stage 4) were mostly associated with ZAP-70+ and CD38+, this is in accordance with previously reported data by [20] who reported that ZAP-70+CD38+ patients were characterized by being associated with advanced Rai stage.

Also, the present study showed that ZAP-70− and CD3 8− patients were characterized by being associated with earlier Rai stage (stage 1 and stage 2). This is also in agreement with the reported data by [20].

It was concluded that, there was a statistically significant difference between Rai stages in the two groups ZAP-70+ and ZAP-70− patients where positive ZAP-70 cases showed higher [20] who observed that ZAP-70 expression was significantly associated with advanced Rai stages as 94.7% of patients expressing ZAP-70 were in stage 3 and 4.

Also, our study revealed that there was a statistically significant difference between Rai stages in the two patients groups CD38 + and CD38− where positive CD38 cases showed higher prevalence of stage 3 and 4 this is in agreement with the previously reported data by [20] who observed that high CD38 expression was associated with advanced Rai staging.

In the present study we concluded that CD38 and ZAP-70 were correlated with each other and advanced stages of CLL. Some patients had indolent disease without complications for many years; others developed progressive disease requiring therapy within a short time after diagnosis. Early treatment of the former could lead to therapy related complications that might compromise their quality of life and/or survival. Defining markers that reliably can stratify patients into groups with good- or poor-risk disease could facilitate clinical trials evaluating the potential benefit of early treatment.

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الملخص العربى

مقدمة:سرطان الدم الليمفاوى المزمن (CLL) هو سرطان الدم الأكثر شيوعا فى المسنين والذى يتميز بتراكم الخلايا الليمفاوية الناجم عن تراكم نسيلى من الخلايا B & CD5 & CD19 فى الدم ونخاع العظام والأجهزة اللمفاوية.

أهم العوامل النذير فى CLL هى المرحلة الطبية، وعلامات الورم من الحمل (على سبيل المثال، ثيميدين كيناز (TK) و–β2 المكروجلوبولين (β2MG) الخلوية التعبير البروتين مثل (CD38) (ZAP70).

الهدف من العمل: لتحديد علامات النذير CD38 و زاب ٧٠ فى المرضى المصريين بسرطان الدم الليمفاوى المزمن من أجل تقسيمها لهم قبل البدء فى أى علاج، وتحديد تأثيرها على نتائج العلاج والبقاء على قيد الحياة.

المواضيع والأساليب:وستجرى الدراسة فى ٢٠ مريضا شخصت حديثا المزمنة مرضى سرطان الدم الليمفاوى زيارة العيادة الخارجية أو هى فى قصر العينى مركز للأورام والطب النووى. وسيتم إدراج عشرين العمر والجنس متطوعين أصحاء للمتطابقة فى الدراسة كمجموعة تحكم.

النتائج: أظهر ١٨ مريضا (٦٠٪) من أصل ٣٠ مستوى عال من ZAP-70 وCD38 فوق قيمة قطع حين المتبقية (٤٠٪) وأظهرت تدنى مستوى ZAP-70 وCD38.

الخلاصة:كان هناك فروق ذات دلالة إحصائية بين انطلاق فى المجموعتين (CD38+/ZAP-70+ وZAP-70–/CD38–). وأظهرت الحالات الإيجابية انتشار أعلى من مراحل الثالث والرابع فى حين أظهرت الحالات السلبية انتشار أعلى من المرحلة الثانية. وفيما يتعلق تحليل البقاء على قيد الحياة، لم يكن هناك فروق ذات دلالة إحصائية بين المجموعتين، ولكن؛ وأظهرت حالات إيجابية أعلى الوسيط وتعنى فترة البقاء على قيد الحياة من الحالات السلبية.