The Incidence of Expression of ZAP-70 and CD38 in Chronic Lymphocytic Leukemia Patients

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ABSTRACT

Background: Chronic Lymphocytic Leukemia (CLL) is the most common chronic lymphoproliferative disorder. ZAP-70 and CD38 are among the prognostic parameters in CLL.

Aim of Study: This study was undertaken to know the prevalence of ZAP-70 and CD38 in the treatment naive patients of CLL seen at the Dammam University Hospital, Saudi Arabia.

Material and Methods: ZAP-70 and CD38 were tested by flow cytometry on peripheral blood samples. ZAP-70 and CD38 positivity was defined as expression on 20% and 30% of CLL cells, respectively. Clinico-hematological profile and its correlation with ZAP-70 and CD38 expression was assessed in consecutive 80 CLL patients.

Results: There were 64 males and 16 females with an age range of 52-75 with a median of 58 years. Sixteen patients (20%) were asymptomatic and diagnosed incidentally. Median Total Lymphocyte Count (TLC) at presentation was 62 X 10^9/L. Rai stage distribution was: Stage 0-6, stage I-20, stage II-36, stage III-5, and stage IV-13. ZAP-70 and CD38 positivity were detected in 20 patients (25%) and 29 patients (36%), respectively. Eleven patients were positive and 34 were negative for both ZAP-70 and CD38 yielding a concordance rate of 56%. There was no statistically significant difference between ZAP-70 and CD38 positivity and negativity with regard to age, sex, Lymphocyte count, lymphadenopathy, organomegaly, and Rai staging.

Conclusion: ZAP-70 and CD38 positivity were detected in 25% and 36%, respectively, with concordance rate of 56%, which is higher than Western literature. There was no correlation of ZAP-70 or CD38 positivity with age, sex, lymphadenopathy, organomegaly, or Rai staging.

Key Words: CD38 – Chronic lymphocytic leukemia – ZAP-70.

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a common leukemic disorder in the West, with an estimated incidence in the United States of 5.17 per 100,000, representing 20% of all mature B-cell neoplasm [1].

CLL patients with advanced stage disease and those who progress from early stage disease are treated with chemotherapy [2]. Overall about 70% of all patients will require therapy during the course of the disease. Although Rai staging system predicts overall survival, it does not predict which patients in the early stages (0-I) will progress and require therapy [3]. 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 [4,5]. In the past few years the focus of research in prognostic factors in CLL has changed from clinical to biological factors. The IgVH mutational status, CD 38 and ZAP-70 expression are few of such markers. Till date the strongest independent prognostic factor for survival in CLL is the presence of somatic mutations of the variable region of the immunoglobulin heavy chain gene [6]. Patients with unmutated IgVH gene have aggressive disease, requiring early treatment and often show poor response to chemotherapy. However, IgVH mutation studies are cost and labor intensive and are not widely available at most centers. Therefore, there was a need for simpler, reliable, and easily standardized surrogate marker, which can substitute IgVH mutation testing. The best studied of these new prognostic parameters have been proteins residing on the cell surface (CD-38) or in the

cytoplasm (ZAP-70), recurring genetic defects in the CLL cells (detected by Fluorescence In-Situ Hybridisation (FISH)), and the mutation status of Ig VH gene. Zeta-associated protein 70 (ZAP-70) is a tyrosine kinase protein normally expressed in T cells and natural killer cells. ZAP-70 has been reported to be expressed preferentially in CLL un-mutated IgVH [7]. The ligation of B-cell receptor on CLL cells that express ZAP-70 is associated with excessive tyrosine phosphorylation [8]. There are several methods used to study the expression of ZAP-70 in patients with CLL; flow cytometric detection of ZAP-70 is relatively reliable and could be placed into more routine use [9]. CD 38 is a 45-kDa, non-lineage 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 [10]. CD 38 also functions as receptor that induces proliferation and increases survival of CLL cells [11]. CD 38 positivity (defined as at least 30% positive cells) is an independent prognostic marker for an unfavorable clinical course in CLL [12]. CD 38 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 [13]. However, it is not a surrogate marker for IgVH mutational status. It has been found that there is significant correlation between CD 38 positivity and intermediate/high modified Rai stages, multiple bulky lymphadenopathy, and splenomegaly [14,15]. There are no much published data available in developing countries about prevalence of ZAP-70 and CD 38 positivity in CLL patients, so this study was undertaken to know the prevalence of CD 38 and ZAP-70 in CLL patients in a developing country and to correlate this with baseline parameters. With longer follow-up we will be able to know the prognostic value of ZAP-70 and CD 38, which will be reported later on.

MATERIAL AND METHODS

Between January 2012 and December 2013, in Dammam University Hospital, consecutive 80 cases of treatment naive CLL patient were selected for this analysis. All cases fulfilled the National Cancer Institute working group criteria for diagnosis of CLL [16]. The protocol was approved by the ethical committee of the hospital. Two milliliters of peripheral venous blood were collected in ethylenediaminetetraacetic acid from each patient after taking informed consent as per guidelines of the ethics committee. A standard whole blood lysis method was used for sample preparation. Briefly, 1×10^6 cells were incubated with preconjugated monoclonal antibodies: CD 38 conjugated to Alexa Fluor 488, CD 19 to phycoerythrin cyanine 5.5 (PE-Cy 5.5), and CD 5 to allophycocyanin (APC), (BD Pharmingen, San Diego, CA, USA) at Room Temperature (RT) in the dark for 20 minutes. Two milliliters of Fluorescence Activated Cell Sorter (FACS) Lyse (BD Pharmingen, San Diego, CA, USA) were added and incubated for 2 hours in the dark at RT followed by incubation for ZAP-70 Phycoerythrin (PE) for 30 minutes (BD Biosciences, San Jose, CA, USA). The cells were then washed and re-suspended in phosphate buffer saline and kept at 4ºC till acquisition. A total of 10.000 events were acquired on a flowcytometer (FACS Canto, BD Biosciences, San Jose, CA, USA) equipped with facility for at least 4-color immunophenotyping and analysis was done using FCS express software version 3.0 (Denovo software, Los Angeles, CA, USA). CLL cells were identified as CD 5+ CD 19+ events and expression of CD 38 and ZAP-70 was evaluated on these gated CLL cells. A cutoff value of 30% to define expression of CD38 and 20% to define expression of ZAP-70 was used Fig. (1).

Statistical analysis:

Quantitative variables were summarized as median and qualitative variables as proportions. Baseline categorical variables were analyzed using Chi-Square test/Fisher's Exact *t*-test. Multivariate logistic regression for independent prognostic value of CD38 and ZAP-70 expression was performed using STATA software version 11.1 (StataCorp, Texas, USA) and $p < 0.05$ was defined as significant.

RESULTS

The median age was 57 years (range 28-90 years). There were 64 males and 16 female patients. Eighteen patients were asymptomatic. Out of 80 patients, 6 (7.5%) were in Rai stage 0, 20 (25%) in Rai stage I, 36 (45%) in Rai stage II, 5 (6.25%) in Rai stage III, and 13 (16.25%) were in Rai stage IV. The median

hemoglobin was 11g/dL with range of 4.7-16 g/dL. The median total leucocytic count was 62×10^9/L and absolute lymphocyte count was 51×10^9/L. The median platelet count was 150×10^9/L. Out of 80 patients, 26 (32.5%) were in Rai stage 0 and I, 36 (45%) were in Rai stage II, 18 (22.5%) were in Rai stage III and IV. CD 38 was positive in 29 (36.25%) of patients. ZAP-70 was positive in 20 (25%) patients. Stage wise distribution of ZAP-70 and CD 38 positive patients is given in (Table 1). Out of 80 patients in whom both ZAP-70 and CD 38 were tested,

11 were concordant ZAP-70+, CD38+ and 34 were ZAP-70-and CD 38-, yielding a concordant rate of 56%. After analyzing ZAP-70 and CD38 as continuous variables no definite correlation was found with age, sex, lymphadenopathy, organomegaly or Rai staging (Table 2). With a median follow-up of 17 months, 11 patients of early stage disease progressed; 3 were ZAP-70 positive, 4 were CD 38 positive, and 4 patients were negative for both. There was no difference between progression free period of ZAP-70 and CD38 positive group.
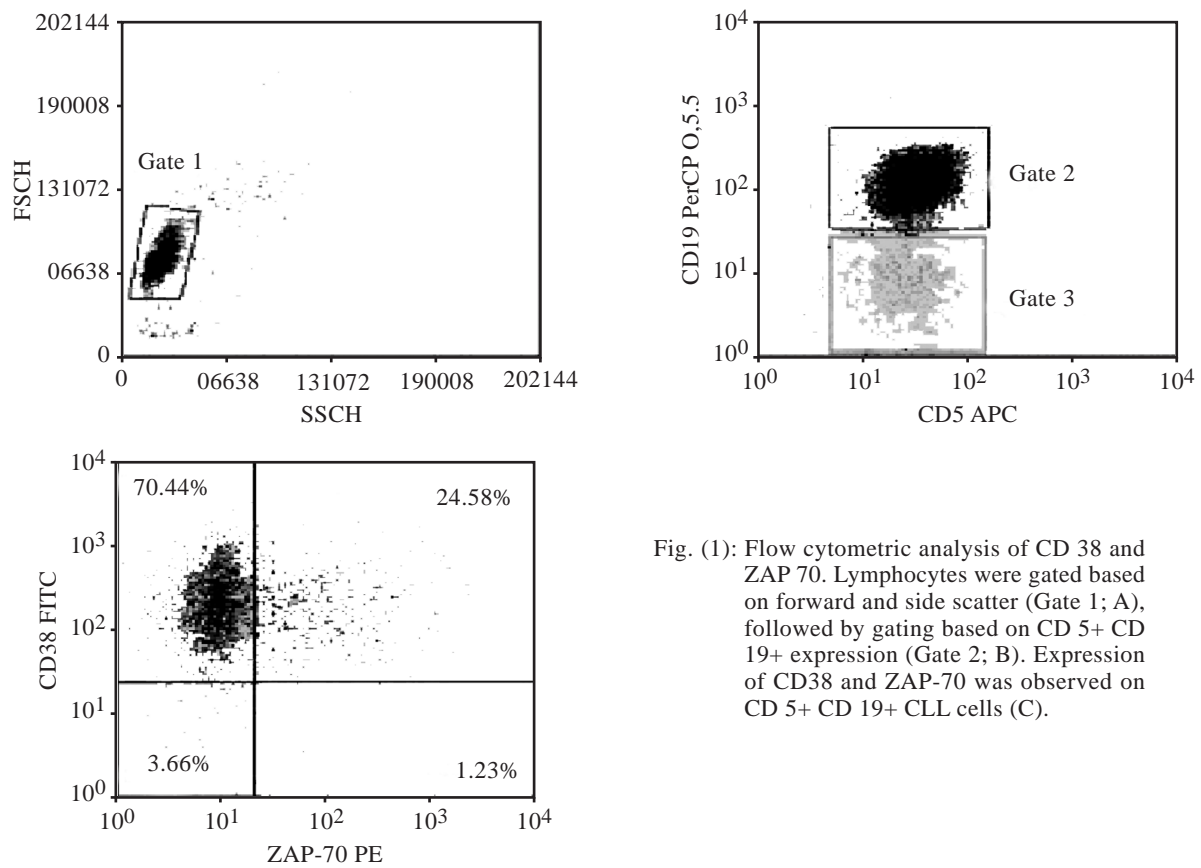

Fig. (1): Flow cytometric analysis of CD 38 and ZAP 70. Lymphocytes were gated based on forward and side scatter (Gate 1; A), followed by gating based on CD 5+ CD 19+ expression (Gate 2; B). Expression of CD38 and ZAP-70 was observed on CD 5+ CD 19+ CLL cells (C).

Table (1): Stage distribution as per ZAP-70 and CD38 in 80 chronic lymphocytic leukemia patients.

	Stade no (%)			
	Rai 0 and I (%)	Rai II (%)	Rai III and IV (%)	*p*-value
ZAP (n=80):				
Positive	9 (11.25)	7 (8.75)	4 (5)	0.249
Negative	17 (21.25)	29 (36.25)	14 (17.5)	
CD 38 (n=80):				
Positive	8 (10)	15 (18.75)	6 (7.5)	0.130
Negative	18 (22.5)	21 (26.25)	12 (15)	

Table (2): Co-relation of ZAP-70 and CD38 with age and base line clinical and laboratory parameters in 80 chronic lymphocytic leukemia patients.

Variables	ZAP-70		CD 38	
	Odds ratio	*p*-value (95% confidence interval)	Odds ratio	*p*-value (95% confidence interval)
Age	0.99	0.843 (0.93-1.06)	1.05	0.110 (0.99-1.11)
Gender (Female)	0.45	0.323 (0.09-2.19)	0.39	0.196 (0.09-1.62)
Hb	1.16	0.456 (0.78-1.72)	0.96	0.813 (0.67-1.36)
TLC	1.00	0.171 (1.0)	1.00	0.205 (1.00)
ALC	1.00	0.213 (1.0)	1.00	0.213 (1.00)
Platelets	0.56	0.396 (0.15-2.14)	1.58	0.451 (0.48-5.24)
Lympadenopathy (No)	0.43	0.212 (0.12-1.61)	1.00	0.997 (0.31-3.27)
Hepatomegaly (No)	1.13	0.875 (0.24-5.33)	1.56	0.486 (0.45-5.44)
Splenomegaly (No)	0.37	0.192 (0.08-1.66)	0.65	0.488 (0.19-2.20)
Stage (Stage 0):				
I	1.11	0.927 (0.11-11.21)	1.36	0.786 (0.15-12.48)
II	0.35	0.370 (0.03-3.52)	3.08	0.310 (0.35-27.02)
III	0.17	0.310 (0.01-5.25)	1.26	0.874 (0.07-22.16)
IV	0.33	0.490 (0.01-7.58)	1.50	0.785 (0.08-27.04)

Hb   : Hemoglobin.
TLC : Total Leukocytic Count.
ALC : Absolute Lymphocyte Count.

DISCUSSION

Rai clinical stage is the most robust and established prognostic factor in CLL. The limitation with this staging system is that a significant percentage of patients with early stage disease will rapidly escalate to advanced disease that requires therapy. Unfortunately, the Rai system is unable to prospectively differentiate the rapidly evolving patient from more stable patients who may not progress for decades. It was found that patients positive for ZAP-70 in early stage disease had a shorter time to therapy, with a median time from diagnosis to initial therapy of 2.9 years, compared with 9.2 years for ZAP-70 negative patients for the same stages [7]. ZAP-70 holds significant promise as a prognostic marker; it was reported to be highly predictive of time to treatment in a large cohort of early stage (Rai 01) and untreated CLL [7,8]. Various studies have reported ZAP-70 positivity in CLL ranging from 36% to 57% (Table 3).

The optimal cutoff for defining ZAP-70 positivity was reported as 20% by various authors [9,14,15,17]. Del Poeta et al., and Hus et al., found significant correlation between high ZAP-70 levels and advanced Rai stage and splenomegaly [12,14]. In this study among the 80 patients 25% (20 patients) were ZAP-70 positive. There was no correlation of ZAP-70 with age, sex, hemoglobin, lymphocyte count, organomegaly or clinical Rai stage. Zeeshan R. et al., [18], said "The frequency of ZAP-70 positivity in B-CLL patients was found to be 13.5%. ZAP-70 positivity was significantly correlated with stage III disease and high absolute lymphocytic count ($p<0.05$). No correlation of ZAP-70 could be established with age and gender ($p>0.05$)".

Various studies have reported CD38 positivity in CLL ranging from 29% to 60% (Table 3). In this study 80 patients were tested and 29% were CD38 positive. In this study 80 patients were tested and 29% were CD38 positive.

Table (3): Comparison of ZAP-70 and CD38 with literature.

Study	No. of patients	ZAP 70%	CD 38%
Crespo et al., [9]	56	57	60
Hus et al., [14]	156	36	33
Schoroer et al., [13]	252	46	29
D'Arena et al., [15]	157	36	29
Present study	80	25	36

ZAP: Zeta-Associated Protein.

The Rai system is based upon the concept that in CLL there is a gradual and progressive increase in the body burden of leukemic lymphocytes, starting in the blood and bone marrow (lymphocytosis), progressively involving lymph nodes (lymphadenopathy), spleen and liver (organomegaly), with eventual compromise of bone marrow function (anemia and thrombocytopenia). In the original series describing the Rai system [19], the stage at the time of initial diagnosis was approximately: Stage 0 (lymphocytosis) 25%, Stages I to II (lymphadenopathy, organomegaly) 50%, Stages III to IV (anemia, thrombocytopenia) 25%. Since this time, some reports have described a shift to earlier stages at initial presentation [20], while others have not [21]. This may reflect changes in the use of "routine" laboratory testing with complete blood counts. In addition, the estimated median survival by stage has improved as new treatments have evolved. In our study, out of 80 patients, 8 (10%) were in Rai stage 0 and I, 15 (18.7%) were in Rai stage II, 6 (7.5%) were in Rai stage III and IV. This differs from early literature because of the low number and short follow-up period of time.

There was no correlation of CD38 with age, sex, hemoglobin, lymphocyte count, organomegaly, or clinical Rai stage. Because of short follow-up this data is not mature to do survival analysis.

Conclusion:

The present study was aimed to study the incidence of two prognostic markers namely ZAP-70 and CD38 positivity in CLL patients. ZAP-70 and CD38 positivity were detected in 20 patients (25%) and 29 patients (36%), respectively, with concordance rate of 56%. There was no correlation of ZAP-70 and CD38 positivity with age, sex, lymphadenopathy, organomegaly, and Rai staging. The low prevalence rate of ZAP-70 and high prevalence of CD38 was due to biology of disease in the Middle East population.

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