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Research Article

ZAP-70 Expression in B-Chronic Lymphocytic Leukemia in Sudanese Patients

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Abstract

Background: Chronic lymphocytic leukemia is the most common form of leukemia in adults. The prognostic impact of ZAP-70 in CLL has been reported in several studies. The aim of conducting this study was to investigate the prevalence of ZAP-70 in Sudanese patients with chronic lymphocytic leukemia attending Khartoum Oncology Hospital.

Materials and Methods: A total of 93 newly diagnosed patients with chronic lymphocytic leukemia were enrolled in this study. Lymphadenopathy and organomegaly were assessed in all participants using clinical examination, chest radiography, and abdominal ultrasound. Full blood count was carried out by an automated hematology analyzer. ZAP-70 was evaluated using flowcytometry on peripheral blood samples. ZAP-70 was defined as positive expression at a cutoff level of 20%.

Results: There were 63 (67.7%) males and 30 (32.3%) females and the median age of the group was 63 years; 68 patients (73.1%) were presented with anemia and 66 (70.9%) had lymphadenopath;y. Majority of our patients 35 (37.6%) were in Rai stage IV. ZAP-70 positivity was detected in 21 patients (22.6%). There was no statistically significant association of ZAP-70 with age, sex, lymphadenopathy, organomegaly, hemoglobin concentration, total white blood cell count, platelet count and Rai staging system (p-value > 0.05).

Conclusion: Only 21 patients (22.6%) were ZAP-70 positive. There was no association between ZAP-70 and the study variables. Further studies to evaluate prognostic role of ZAP-70 in Sudanese patients with chronic lymphocytic leukemia are recommended.

Keywords: ZAP-70, chronic lymphocytic leukemia, flowcytometry

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1. Introduction

Chronic lymphocytic leukemia (CLL) is characterized by a heterogeneous clinical course [1] with survival times ranging from months to decades; this heterogeneity reflects the biological diversity of CLL [2]. Neoplastic cells express Certain markers that can help to predict the prognosis of patients with CLL [3, 4]. The detection of these markers provides a vital tool that can stratify patients into groups with good or poor prognosis [5].

The traditional prognostic parameters (clinical stage, pattern of bone marrow infiltration, lymphocyte doubling time, beta-2 microglobulin levels, and lactate dehydrogenase level) are valuable but less accurate in prediction of disease progression [6, 7].

Last years, research in prognostic factors in CLL has focused on biological factors such as the variable region of the immunoglobulin heavy chain gene (IgVH) mutational status, CD38 and ZAP-70 expression. The strongest independent prognostic factor in CLL is the presence of IgVH somatic mutations [8]. However, IgVH mutation studies are not cost effective, technically demanding and are not available at most centers. Therefore, there is a need for surrogate marker that is reliable, easily standardized and more suitable for application in clinical laboratories [9].

The zeta-chain-associated protein kinase-70 (ZAP-70) was first described in 2003 [5] and is present in normal T-cells, NK-cells, and not present in normal B-cells. Published reports demonstrated a strong association between overexpression of ZAP-70 and unmutated IGHV genes in CLL. Also, low expression of ZAP-70 is associated to mutated IGHV genes [5, 10, 11] and time to disease progression and overall survival (OS) [5, 10–13].

Although there has a consensus on the prognostic significance of ZAP-70, the detection method of ZAP-70 at various laboratories has been a source of discussion [14]. Several methods are used to study ZAP-70 expression in CLL but flowcytometry remains the most suitable, reliable and applicable method into routine use [10].

Crespo et al. [15] and Rassenti et al. [11] demonstrated that patients with more than 20% ZAP-70 positive cells have higher risk of disease progression and lower survival rates compared with those with less than 20% ZAP-70-positive cells, suggesting that ZAP-70 might be more indicative of worse prognosis compared with the mutational status of the *IGHV*.

In this study, the authors aimed to determine the frequency of ZAP-70 among Sudanese patients with chronic lymphocytic leukaemia and to associate ZAP-70

expression to age, sex, lymphadenopathy, organomegaly, haemoglobin concentration, TWBCs count, platelet count, and modified Rai clinical staging system.

2. Materials and Methods

2.1. Patients

A total of 93(69.7% males and 30.3% females) newly diagnosed (based on CBC and immunophenotyping), untreated B-CLL patients, attending the Khartoum Oncology Hospital during the period from September 2016 to February 2017, were enrolled in this cross-sectional study. Patients were diagnosed as CLL, in accordance with the International CLL Workshop Criteria [16] and the Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia [17]. Also, all the patients were staged according to the Rai staging system [18]; 3 ml of peripheral blood (PB) was collected in EDTA tubes from each patient according to the standard protocol. The PB samples were processed within 6–24 h of collection, preserved at room temperature (22–24°C). Clinical and demographic data were collected in a predesigned questionnaire. This study was approved by the ethical committee of the Sudan Medical Specialty Board (SMSB) and an informed consent was obtained from all patients before sampling in accordance to the guidelines and requirements of the ethical committee.

3. Methods

3.1. Clinical examination

Each patient was subjected to a history taking and physical examination that was performed to determine the diameters of lymph nodes. Chest X-ray and abdominal ultrasound were used to evaluate the size of the liver and spleen.

3.2. Complete Blood Count (CBC)

CBC was carried out using automated cell counter (Sysmex XE-2100^{TM}).

3.3. Immunophenotyping (IPT)

IPT was carried out to confirm the diagnosis of CLL using the following monoclonal antibodies (Mo Ab) (Beckman Coulter); CD45, CD5, CD19, CD20, CD22, CD23, kappa and

lambda light chains, FMC7, CD79b. A marker was considered positive at a cutoff level of 20%.

3.4. Flowcytometric analysis of intracellular ZAP-70 expression

Four color flowcytometer (COULTER EPICS XL-MCLTM Flowcytometer – Miami, Florida – USA) with SYSTEM II software was used to detect and measure the intracellular ZAP70 expression in PB samples according to the following protocol; First PerFix-nc a fixation and permeabilization kit was used as follows: Pipette 50 µL of blood sample into the bottom of each appropriately labeled tube. Add 5 µL of the FixativeReagent to each tube. Vortex immediately and incubate for 15 min at room temperature (18–25˚C). Vortex the fixed specimen again and add 300 µL of the Permeabilizing Reagent to each tube; vortex immediately. Then, immediately add to each tube 20 µl ZAP-70 Isoclonic Control. Vortex immediately and incubate for 10 min at room temperature (18–25˚C). Add to each tube 20 µL ZAP-70-PE (PN B57658). Vortex immediately and incubate for 45 min at room temperature protected from light. Add 3 mL of the Final 1 X Reagent (prepared from the 10 X concentrated Final Solution) to each tube; vortex immediately. Pellet the cells at 500 x g for 5 minutes, and completely discard the supernatant by aspiration. Re-suspend the cell pellet in 0.5 mL of the same Final 1X Reagent; the sample is now ready for acquisition on a flowcytometer. The ZAP-70 expression was considered positive at a cutoff level of 20%.

3.5. Data analysis

The collected data were analyzed using the software program of the Statistical Package for Social science for Windows (SPSS), version 19. Quantitative variables were summarized as mean and median and qualitative variables as percentage. Categorical variables were analyzed using Chi-Square test. P value less than 0.05 was defined as significant.

3.6. Results

A total of 93 CLL patients (63 (67.7%) males and 30 (32.3%) females) were involved in this study with a mean (± SD) age 62.29 ± 11.68, median 63 years (range: 36–95). According to Rai staging system, 35 (37.6%) patients were in Stage IV, 28 (30.1%) patients were in Stage III, 17 (18.3%) patients were Stage II, 9 (9.7%) patients were

in Stage I, 4 (4.3%) patients were Stage 0; 66 (71.0%) patients presented with lymphadenopathy, 50 (53.8%) with splenomegaly, 23 (24.7%) with hepatomegaly. Anemia was detected in 68 (73.1%) patients and thrombocytopenia in 42 (45.2%) patients **(Table 1)**. Immunophenotyping results revealed characteristic CLL immunophenotype.

We found that the mean (±SD) expression of ZAP70 was 17.06 ± 21.89, with a median of 8.3 (range: 0.03–85.5%). Out of 93 patients, only 21 (22.6%) patients were positive for ZAP70 expressions at cutoff levels of 20%, whereas 72 (77.4%) patients were negative. No significant association was observed between ZAP70 expressions at cutoff levels of 20% and the study variables (Age, Sex, Lymphadenopathy, organomegaly, Hb concentration, TWBCs count, Platelets count, and Rai staging system) (p-value > 0.05) (**Table 2**).

Table 1: Demographic, clinical and laboratory characteristics of patients.

Parameter	Frequency No. (%)	Parameter	Frequency No. (%)
Age		**Hemoglobin (g/dl)**	
Mean ± SD	62.29 ± 11.68	Low	68 (73.1%)
Median (range)	63 (36–95)	Normal	25 (26.9%)
≥ 60	61 (65.6%)	**Leukocyte count ($\times 10^9$/L)**	
< 60	32 (34.4%)	< 50	27 (29.0%)
Sex		≥ 50	66 (71.0%)
Male: Female Ratio	1:0.48	**Platelets count ($\times 10^9$/L)**	
Male	63 (67.7%)	< 150	42 (45.2%)
Female	30 (32.3%)	≥ 150	51 (54.8%)
Lymphadenopathy		**Rai staging system**	
Present	66 (71.0%)	Stage 0	4 (4.3%)
Absent	27 (29.0%)	Stage I	9 (9.7%)
Splenomegaly		Stage II	17 (18.3%)
Present	50 (53.8%)	Stage III	28 (30.1%)
Absent	43 (46.2%)	Stage IV	35(37.6%)
Hepatomegaly		**ZAP-70 Expression**	
Present	23 (24.7%)	Positive	21 (22.6%)
Absent	70 (75.3%)	Negative	72 (77.4%)

4. Discussion

ZAP-70 has a great future as a prognostic marker as it has been found that ZAP-70 is highly predictive of time to treatment in a large cohort of early-stage (Rai 0-1) and untreated CLL [11, 19]. The optimal cutoff for ZAP-70 positivity was defined by many authors as 20% [10, 12, 20, 21]. We found that 21 (22.6%) patients were positive for

Table 2: Association of ZAP-70 expression with the study variables.

Variables	Zap-70 Frequency No. (%)		P-value
	ZAP-70 positive	ZAP-70 negative	
Age			
≥ 60	11 (52.4%)	50 (69.4%)	0.15
< 60	10 (47.6%)	22 (30.6%)	
Sex			
Male	14 (15.1%)	49 (52.7%)	0.91
Female	7 (7.5%)	23 (24.7%)	
Lymphadenopathy			
Present	15 (16.1%)	51 (54.8%)	0.96
Absent	6 (6.5%)	21 (22.6%)	
Splenomegaly			
Present	12 (12.9%)	38 (40.9%)	0.83
Absent	9 (9.7%)	34 (36.5%)	
Hepatomegaly			
Present	5 (5.4%)	18 (19.4%)	0.85
Absent	16 (17.2%)	54 (58.0%)	
Hemoglobin concentration (g/dl)			
Low	15 (16.1%)	53 (57.0%)	0.84
Normal	6 (6.5%)	19 (20.4%)	
TWBCs count $\times 10^9$			
< 50	5 (5.4%)	22 (23.7%)	0.55
≥ 50	16 (17.2%)	50 (53.7%)	
Platelet count $\times 10^9$			
< 150	9 (9.7%)	33 (35.5%)	0.81
≥ 150	12 (12.9%)	39 (41.9%)	
Rai staging system			
Stage 0	1 (1.1%)	3 (3.2%)	0.88
Stage I	1 (1.1%)	8 (8.6%)	
Stage II	3 (3.2%)	14 (15.1%)	
Stage III	7 (7.5%)	21 (22.6%)	
Stage IV	9 (9.6%)	26 (28.0%)	

ZAP70 expressions at cutoff levels of 20% and 72 (77.4%) patients were negative. Many published reports indicated ZAP-70 positivity in CLL ranging from 25% to 57% as presented in **Table 3**; our result is slightly lower.

Our results revealed that there was no significant association of ZAP-70 with age, sex, lymphadenopathy, organomegaly, hemoglobin concentration, TWBCs count, Platelets count, and Rai staging system ($p > 0.05$). Our findings go in line with a study done by Gogia et al. from India who reported no association of ZAP-70 positivity with

age, sex, lymphadenopathy, organomegaly, and Rai staging system [9]. A study by Del Poeta et al. and Hus et al. indicated a significant correlation between high ZAP-70 levels and advanced Rai stage and splenomegaly [20 22]. This is in disagreement with our results. On the other hand, El-Kinawy et al. reported that ZAP-70 expression was significantly associated with advanced Rai stages 3 and 4, while it was negatively correlated to Hb levels and platelet counts [23].

Crespo et al. [10] found that at a cutoff of 20%, ZAP70 positivity clearly separated CLL patients into two groups; those with < 20% ZAP70 had increased survival time and decreased chance of disease progression.

In most laboratories, ZAP70 is considered positive when at least 20% of the CLL-cells have a signal that is greater than the background control signal. Subjectivity in these methods can lead to variable results among research laboratories. To overcome this subjectivity, it has been suggested that mean fluorescence intensity (MFI) values from CLL-cells and background T lymphocytes should be measured rather than calculating the percentage of positive cells [24]. Rossi et al [24] reported that when using MFI values and calculating the ratio of T-cells to B-cells, those patients with a ratio lower than 3 had a shorter time-to-treatment than ZAP70-negative CLL patients and those estimated to be ZAP70-positive by the T-cell percentage method only [25].

Unfortunately, prognostic impact of ZAP70 could not be investigated in our study due to lack of follow-up.

Contrast of our results with other workers may be contributed to by many factors such as biology of disease in the Sudanese population, sample sizes, and sensitivity of method.

Table 3: Comparison of ZAP 70 with literature.

Study	Year	No. of Patients	ZAP 70%
Crespo et al. [10]	2003	56	57
Schoroer et al. [26]	2005	252	46
Hus et al. [24]	2006	156	36
D'Arena et al. [21]	2007	157	36
Gogia et al. [9]	2013	80	25
Present study	2017	93	22.6

5. Conclusion

The present study was aimed to study the prevalence of ZAP-70 in CLL patients and to relate them to the study variables (age, sex, lymphadenopathy, organomegaly,

hemoglobin concentration, TWBCs count, Platelets count, and Rai staging system). ZAP-70 positivity was detected in 21 patients (22.6%). There was higher frequency of high-risk group among study population. There was no significant association of ZAP-70 positivity with the study variables. The prognostic value of ZAP-70 should be tested in the setting of a controlled prospective trial. Further studies are strongly recommended to develop a standardized flowcytometry protocol using MFI and T/B ratio method to verify the prognostic impact of ZAP70.

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Conflict of Interests

The authors declare that they have no competing interests.

References

[1] Rozman, C. and Montserrat, E. (1995). Chronic lymphocytic leukaemia. *The New England Journal of Medicine*, vol. 333, pp. 1052–1057.

[2] Rodringuez-Vicente, A. E., Diaz, M. G., and Hernandez-Rivas, M. J. (2013). Chronic lymphocytic leukemia: A clinical and molecular heterogenous disease. *Cancer Genetics*, vol. 206, no. 3, pp. 49–62.

[3] Stevenson, F. K. and Caligaris-Cappio, F. (2004). Chronic lymphocytic leukaemia: Revelations from the B-cell receptor. *Blood*, vol. 103, pp. 4389–4395.

[4] Chiorazzi, N., Rai, K. R., and Ferrarini, M. Chronic lymphocytic leukaemia. (2005). *The New England Journal of Medicine*, vol. 352, pp. 804–815.

[5] Binet, J. L., Caligaris-Cappio, F., Catovsky, D., et al. (2006). Perspectives on the use of new diagnostic tools in the treatment of chronic lymphocytic leukemia. *Blood*, vol. 107, pp. 859–861.

[6] Molica, S. (1991). Progression and survival studies in early chronic lymphocytic leukemia. *Blood*, vol. 78, pp. 895–899.

[7] Montserrat, E., Sanchez-Bisono, J., Vinolas, N., et al. (1986). Lymphocyte doubling time in chronic lymphocytic leukaemia: Analysis of its prognostic significance. *British Journal of Haematology*, vol. 62, pp. 567–575.

[8] Hamblin, T. J., Davis, Z., Gardiner, A., et al. (1999). Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*, vol. 94, pp. 1848–1854.

[9] Gogia, A., Sharma, A., Raina, V., et al. (2013). Prevalence of ZAP-70 and CD 38 in Indian chronic lymphocytic leukemia patients. *Indian Journal of Cancer*, vol. 50, no. 4, pp. 333–336.

[10] Crespo, M., Bosch, F., Villamor, N., et al. (2003). ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *The New England Journal of Medicine*, vol. 348, pp. 1764–1775.

[11] Rassenti, L. Z., Huynh, L., Toy, T. L., et al. (2004). ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *The New England Journal of Medicine*, vol. 351, pp. 893–901.

[12] Orchard, J. A., Ibbotson, R. E., Davis, Z., et al. (2004). ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. *Lancet*, vol. 363, pp. 105–111.

[13] Herishanu, Y., Kay, S., Rogowski, O., et al. (2005). T-cell ZAP-70 overexpression in chronic lymphocytic leukemia (CLL) correlates with CLL cell ZAP-70 levels, clinical stage and disease progression. *Leukemia*, vol. 19, pp. 1289–1291.

[14] Nabhan, C., Raca, G., and Wang, Y. L. (October 2015). Predicting prognosis in chronic lymphocytic leukemia in the contemporary era. *JAMA Oncology*, vol. 1, no. 7, pp. 965–974.

[15] Ghielmini, M., Vitolo, U., Kimby, E., et al. (2013). Panel members of the 1st ESMO consensus conference on malignant lymphoma. ESMO Guidelines consensus conference on malignant lymphoma 2011 part 1: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL). *Annals of Oncology*, vol. 24, no. 3, pp. 561–576.

[16] Cheson, B. D., Bennett, J. M., Grever, M., et al. (1996). National Cancer Institute-sponsored working group guidelines for chronic lymphocytic leukemia: Revised guidelines for diagnosis and treatment. *Blood*, vol. 87, pp. 4990–4997.

[17] Hallek, M., Cheson, B. D., Catovsky, D., et al. (2008). Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: A report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*, vol. 111, pp. 5446–5456.

[18] Rai, K. R., Sawitsky, A., Cronkite, E. P., et al. (1975). Clinical staging of chronic lymphocytic leukemia. *Blood*; vol. 46, no. 2, pp. 219–234.

[19] Del Principe, M. I., Del Poeta, G., Buccisano, F., et al. (2006). Clinical significance of ZAP 70 protein expression in B cell Chronic Lymphocytic Leukemia. *Blood*, vol. 108, pp. 853–861.

[20] Hus, I., Podhorecka, M., Bojarska-Junak, A., et al. (2006). The clinical significance of ZAP70 and CD 38 expression in B cell chronic lymphocytic leukemia. *Annals of Oncology*, vol. 17, pp. 683–690.

[21] D'Arena, G., Tarnani, M., Rumi, C., et al. (2007). Prognostic significance of combined analysis of ZAP 70 and CD 38 in chronic lymphocytic leukemia. Am J Haematol;82:787-91.

[22] Del Poeta, G., Maurillo, L., Venditti, A., et al. (2001). Clinical significance of CD 38 expression in chronic lymphocytic leukemia. *Blood*, vol. 98, pp. 2633–2639.

[23] El-Kinawy, N. S., Sharaf, H. M., and Abd El-Hamid, M. (2012). Prognostic significance of del 17p, ZAP-70 and CD38 as independent indicators for B-CLL: Correlation to response to treatment and disease outcome. *The Egyptian Journal of Medical Human Genetics*, vol. 13, pp. 173–181.

[24] Rossi, F. M., Del Principe, M. I., Rossi, D., et al. (2010). Prognostic impact of ZAP-70 expression in chronic lymphocytic leukemia: Mean fluorescence intensity T/B ratio versus percentage of positive cells. *Journal of Translational Medicine*, vol. 8, no. 23.

[25] Catherwood, M. A., Matthews, C., Niblock, R., et al. (2006). ZAP-70 mRNA quantification in B-cell chronic lymphocytic leukaemia. *European Journal of Haematology*, vol. 76, no. 4, pp. 294–298.

[26] Schroers, R., Griesinger, F., Trumper, L., et al. (2005). Combined analysis of ZAP 70 and CD 38 expression as a predictor of disease progression in B cell chronic lymphocytic leukemia. *Leukemia*, vol. 19, pp. 750–758.