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Estimation of zeta-chain-associated protein 70, interleukin-6 and interleukin-10 levels in sera of Iraqi newly diagnosed chronic lymphocytic leukemia

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Abstract:

BACKGROUND: Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world. The zeta-chain-associated protein (ZAP-70) an intracellular tyrosine kinase which play an important role in T-cell receptor signaling, natural killer cell activation, and early B-cell development. Interleukin-10 (IL-10) production has strong immunosuppressive effects through inhibition of Th1 type cytokines. IL-6 is a pleiotropic cytokine produced by a variety of cell types, including normal hematopoietic cells, and lymphocytes.

OBJECTIVES: The objective of this study is to assess the level of ZAP-70, IL-10, and IL-6 in CLL and to correlate these levels with prognosis.

MATERIALS AND METHODS: A prospective cohort study carried out at the National Center of Hematology from October 2013 to September 2015. Eighteen patients with newly diagnosis of CLL compared to 19 apparently healthy controls were also involved in this study. ZAP-70 was measured by while IL-10 and IL-6 were measured using serological methods including the enzyme-linked immunosorbent assay.

RESULTS: ZAP-70 range between (24% and 90%) with a mean of 46.89 ± 19.15 , two patients out of 18 were negative (<24%) for ZAP-70 as compared with control. The levels of IL-6 in the serum of untreated patients with CLL were increased in compared with healthy control (2.53 ± 1.98), ranged between (0.12 and 6.94) pg/ml. The range of the IL-10 expression of the untreated CLL patients was between (the lowest positive value (212 pg/ml) and which is the highest positive value (987 pg/ml) with a mean 614 ± 301 pg/ml, with all the morphologically diagnosed CLL cases show positive expression for IL-10.

CONCLUSION: ZAP-70 level was higher in CLL patients than control and immunochemotherapy can normalize this which indicated good response to treatment. On the other hand, IL6 and IL10 were also higher in patients with CLL but not affected by therapy.

Key words:

Chronic lymphocytic leukemia, interleukin-10, interleukin-6, zeta-chain-associated protein 70

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Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world accounting for 40% of all leukemia characterized by the clonal proliferation and accumulation of mature B-lymphocytes. It affects mainly elderly patients as the median age of diagnosis is about 72 years and the male to female ratio is 2:1. Rai and Binet staging systems are used for predicting CLL patients' outcome. The symptoms and signs of the disease arise from a clonal excess of B-cells caused mainly by defects that prevent programmed cell death (apoptosis).^[1,2]

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CLL is a disease in which mature lymphocytes become cancerous and gradually replace normal cells as functionally incompetent lymphocytes in the blood, bone marrow, liver, spleen, and lymph nodes as a result of prolonged lifespan with impairment of normal apoptosis in CLL due to overexpression of the Bcl-2 gene product, which is an anti-apoptotic protein.^[3,4] CLL is characterized by the progressive accumulation of mature, monoclonal CD5⁺, CD19⁺, and CD23⁺ B-lymphocytes in the peripheral blood, lymph nodes, spleen, and bone marrow.^[5]

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The common chromosome abnormalities with CLL patients are deletion of 13q14, trisomy 12, deletions at 11q22-q23, NOTCH1, and structural abnormality of 17p involving the p53 gene.^[6,7] The zeta-chain-associated protein of 70 kD (ZAP-70), an intracellular tyrosine kinase which plays an important role in T-cell receptor signaling, natural killer cell activation, and early B-cell development ZAP-70 in B-cells is used as a prognostic marker in identifying different forms of CLL.^[8] ZAP-70 protein is not expressed in most normal mature B-cells but is expressed in various B- and T-cell lymphomas.^[9,10] In previous studies, ZAP-70 protein expression measured by flow cytometry (FCM) was associated with unmutated immunoglobulin heavy chain variable region genes,^[11] that participates in early B-cell differentiation and a prognostic factor in CLL.^[12,13] In a larger series of patients have shown that an increased expression of ZAP-70 by CLL is a more significant predictor of need for treatment than the presence of an unmutated IgVH gene. Moreover, the expression of ZAP-70 appears to be constant over time.^[14] Interleukin-6 (IL-6) is of special interest in B-CLL because this cytokine acts as a B-cell stimulatory factor (BSF). The production of IL-6 *in vitro* varies significantly among patients with different stages of CLL.^[15] Cytokines such as tumor necrosis factor TNF- α , IL-2, IL-4, IL-6, IL-8, IL-10, and IFN- α have been proposed to play a role in the activation, growth, and apoptosis of leukemic B-cells. Plasma levels of IL-6 varies significantly among patients with different stages of CLL.^[16] Cytokines are low molecular weight (<80 kD) glycoproteins produced by a number of cell types, predominantly leukocytes that regulate immunity, inflammation, and hematopoiesis. They are produced from various sources during the effector phases of immune responses and regulate a number of physiological and pathological functions including innate immunity, acquired immunity, and inflammatory responses.^[17] In CLL, leukemic cells do not appear to be the source of IL-6 and IL-10. In regard to survival, some investigators suggest that IL-6 and IL-10 prevents apoptotic death of CLL cells.^[16,18] IL-6 is a protein of 26 KD, with 183 amino acid forming the mature protein; its coding gene located on chromosome no. 7. IL-6 is a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, normal hematopoietic cells, and lymphocytes. IL-6 is an IL-1 inducible and represents the best marker of biologically active IL-1.^[19,20] IL-6 is of special interest in B-CLL because this cytokine acts as a BSF-IL, mediates B-cell differentiation and can stimulate the growth of B-cell lymphoid malignancies such as myeloma. In contrast, it has been proven that IL-6 is also able to inhibit TNF- α induced proliferation of B-cells from CLL patients.^[16,21] IL-10 is a protein of 35 kD. It is produced by several T-cell subpopulations, such as Th2, NK cells, and a variety of cell type, including macrophages, dendritic cells, and B-cells. IL-10 production has strong immunosuppressive effects via inhibition of Th1 type cytokines, including interferon-gamma (IFN- γ) and IL-2, secretion of IL-10 from Treg cells, macrophages and other leukocytes followed by subsequent binding to IL-10 receptors on macrophages and dendritic cells has been linked to a reduced antigen presentation and an increased T-cell energy.^[22] In addition, IL-10 has been demonstrated to be effective in decreasing the levels of some proinflammatory cytokines (IL-2, IL-6, IL-1 β , IL-12, GM-CSF TNF- α , and IFN- γ). IL-10 prevents apoptotic death of CLL cells, whereas others suggest that it enhances them.^[23,24]

Materials and Methods

This study was conducted on 18 adult Iraqi patients referred to National Center of Hematology / Al-Mustansiriyah University. The period extending from October 2013 to September 2015. The diagnosis of CLL based on physical examination, morphological assessment, of peripheral blood films, and bone marrow smear by aspirate examination, as well as flow cytometric immunophenotypic profile.

The treatment given to CLL patients is consist of a combination therapy (rituximab, fludarabine, and cyclophosphamide [R-FC]).

Immunophenotyping

Principle of the assay

In this study, ZAP-70 expression was investigated by using fully equipped desktop four-color FCM. CyFlow Cube features a modular optical concept. This allows using different lasers as light sources. The CyFlow Cube allows easy optimization of the optics for any application by simple exchange of optical filters and mirrors. Data acquisition, instrument control, and data analysis are controlled and performed by the CyView software (Sysmex Partec GmbH Münster, Germany).

Antibody labeling (PARTEC CyFlow®)

Antibody labeling was done by mixing 100 μ l of whole blood with conjugated antibodies (10 μ l) in a test tube, mixed thoroughly, incubated for 15 min in the dark at room temperature.

Leukocyte fixation

For leukocyte fixation, 100 μ l of reagent A was added and mixed thoroughly and incubated for 10 min in the dark.

Erythrocyte lysis

FCM data were analyzed in bivariate plots of two- or three-color analyses with the application of electronic gates based on the scatter characteristic of cells. The measurement of the intensity of staining of cells by FCM provides an absolute value for the light intensity. It measurement is performed by comparing cell fluorescence with an external standard using different commercially available beads in kits, which usually comprise two tubes.

Interleukin-6 detection

Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-6 has been precoated onto a microplate. Standards and samples are pipetted into the wells, and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped, and the intensity of the color is measured.

Assay procedure (as manufacture's instruction)

Before carrying out the assay procedure, the kit was left at room temperature (22°C) for 30 min to equilibrate, as suggested

by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

- One hindered microliter Assay Diluent RD1W was added to each well
- Serial concentrations (0, 3.13, 6.25, 12.5, 25, 50, and 100) pg/ml of the standard was made using the assay diluent after reconstitution of standard vial with 1 ml of diluent water
- An aliquot (100 µl) of the standard or serum sample was added to the well. After that, the well was mixed, and the plate was covered and incubated for 2 h at room temperature
- The wells were washed with four cycles of washing using the washing solution, with the aid of a microtiter plate washer
- 200 µL of IL-6 Conjugate was added to each well. Then, covered with a new adhesive strip and incubated for 2 h at room temperature
- The washing step was repeated (Step IV)
- An aliquot (200 µl) of substrate reagent was added to each well, and the plate was covered and incubated in dark for 20 min at room temperature
- An aliquot (50 µl) of stop solution was added to each well, and the absorbance was read at a wavelength of 570 nm using enzyme-linked immunosorbent assay (ELISA) reader within 30 min after stopping the reaction.

Interleukin-10 detection (R and D Systems, USA)

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-10 has been precoated onto a microplate. Standards and samples are pipetted into the wells, and any IL-10 present is bound by the immobilized antibody after washing away any unbound substances, an enzyme-linked monoclonal antibody specific for IL-10 is added to the wells. Following a wash to remove any unbound antibody – Enzyme reagent a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped, and the intensity of the color is measured.

Assay procedure

Before carrying out the assay procedure, the kit was left at room temperature (22°C) for 30 min to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

- Fifty microliter Assay Diluent RD1W was added to each well
- Serial concentrations (0, 7.8, 15.6, 31, 2, 62.5, 125, 250, and 500) pg/ml of the standard was made using the assay diluent after reconstitution of standard vial with 1 ml of diluent water
- An aliquot (200 µl) of the standard or serum sample was added to the well. After that, the well was mixed, and the plate was covered and incubated for 120 min at room temperature
- The wells were washed with four cycles of washing using the washing solution, with the aid of a microtiter plate washer
- An aliquot (200 µl) of conjugate solution was added to each well, and the plate was covered and incubated for 120 min

at room temperature

- The washing step was repeated (Step IV)
- An aliquot (200 µl) of substrate reagent was added to each well, and the plate was covered and incubated in dark for 30 min at room temperature.

An aliquot (50 µl) of stop solution was added to each well, and the absorbance was read at a wavelength of 450–630 nm using ELISA reader within 30 min after stopping the reaction.

Results

Eighteen newly diagnosed CLL patients (12 males and 6 females), with a mean age of (55 ± 12) year, range of (40–80) year. According to the Binet staging system for CLL, (10) patients were in Stage B and (8) in Stage C. Nineteen (19) patients, apparently healthy controls were also enrolled in this study. They included (9) males and (10) females, with a mean age of (61 ± 17) year and an age range of (30–90) year. Regarding demographic characteristics, the highest age incidence was between age groups (50–59) years and (60–69) years, as approximately (38.9%) and (27.8%) of the incidence of the disease occurred at each of those age groups respectively as shown in Table 1 and Figure 1. Distribution of the patients according to Binet staging is presented in Table 2 which showed that the highest percentage of the patients in this study (55.6%) fell within Binet Stage B of the CLL patients which is considered the intermediate or moderate risk stage in CLL patients, while the lowest percentage of the patients (44.4%) fell within Binet Stage C of the CLL patients which is considered the most advanced stage in CLL patients. Lymphocytes of the newly diagnosed CLL patients expressed significantly increased expression of ZAP-70 compared to healthy controls [Table 3; Figure 2].

Table 1: Age distribution of newly diagnosed chronic lymphocytic leukemia patients of the present study

	Age group (years)					Total, n (%)
	40-49	50-59	60-69	70-79	80-89	
Number of CLL cases (%)	4 (22.2)	7 (38.9)	5 (27.8)	1 (0.05)	1 (0.05)	18 (100)

CLL=Chronic lymphocytic leukemia

Table 2: Distribution of chronic lymphocytic leukemia patients according to Binet staging

	Binet staging		Total, n (%)
	B	C	
CLL cases (%)	10 (55.6)	8 (44.4)	18 (100)

CLL=Chronic lymphocytic leukemia

Table 3: Zeta-chain associated protein-70 in control and newly diagnosed (untreated and treated) chronic lymphocytic leukemia patients: A follow-up study (4.5 months)

Parameters	Control (n=19)	Newly diagnosed CLL patients	
		Untreated (n=18)	Treated (4.5 months), n=14
ZAP-70	7.09±8.32 ^a	46.89±19.15 ^b	9.84±12.78 ^a

Values are expressed as mean±SD. Values with different letters within each parameter are significantly different ($P < 0.05$). CLL=Chronic lymphocytic leukemia, SD=Standard deviation, ZAP-70=Zeta-chain associated protein-70

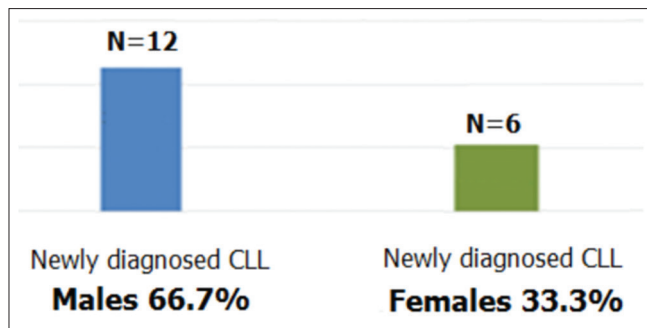


Figure 1: Gender distribution of newly diagnosed chronic lymphocytic leukemia patients

Discussion

CLL is the most common type of leukemia in the Western world accounting for 40% of all leukemia characterized by the clonal proliferation and accumulation of mature B-lymphocytes. It affects mainly elderly patients as the median age of diagnosis is about 72 years and the male to female ratio is 2:1.

The total CLL patients enrolled in the present study were composed of 12 males (66.7%) and 6 females (33.3%) with a higher incidence in male than in female (ratio 2:1). Some studies reported that the ratio of the incidence of male to female (2.1:1) and the highest incidence occurs also frequently in middle age. Others reported that the incidence of CLL increases with age, and the median age at presentation is (65–70) years with a male-predominant disease, although the reason for this difference is not known.^[25,26] Regarding Binet staging, this study found that all patients were in stage B and C which is similar to that reported by Rawstron *et al.*^[27] Clinical staging systems developed by Rai *et al.* (1975) and Binet *et al.* (1981) were able to predict long-term survival of patients with CLL, but have failed to predict specific disease course in patients with early-stage CLL. Advances in the identification and understanding of genomic and molecular markers are helping better predict disease progression and survival.^[6,28]

The zeta-chain-associated protein of 70 kD

In regards to the ZAP-70% range between (24% and 90%) with a mean of 46.89 ± 19.15 , two patients out of 18 were negative (<24%) for ZAP-70 as compared with control. ZAP-70 positivity (>30%) for previously untreated and asymptomatic patients is associated with a more unfavorable median survival (6–10 years), while a negative ZAP-70% is associated with a median survival of more than 15 years.^[12,29] Tsimberidou *et al.* demonstrated that the current treatments for CLL (R-FC) induce apoptosis in CLL cells but lead to significant immune-suppression and patients often develop drug resistance.^[30] Other study on CLL demonstrated that the higher levels of ZAP-70 expression are associated with shorter time to treatment and poorer survival. However, the routine clinical measurement of ZAP-70 expression is difficult and non-standardized.^[12]

Interleukins

By the same token, plasma level of IL-6 and IL-10 were significantly elevated in newly diagnosed CLL patients compared to controls as shown in Table 4. The levels of IL-6 in the plasma of untreated patients with CLL were increased

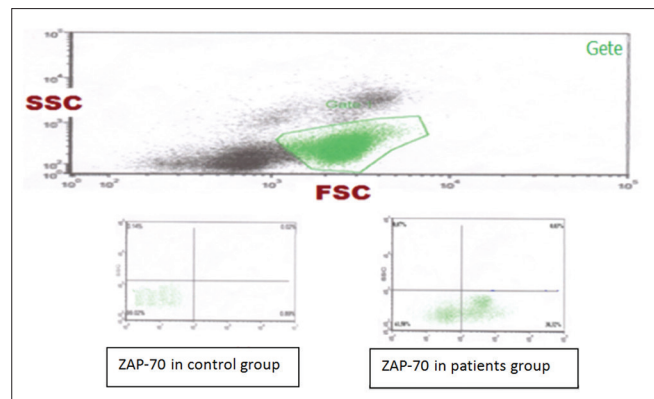


Figure 2: The forward and side scatter light signals of zeta-chain associated protein-70 in patients with chronic lymphocytic leukemia and healthy control

Table 4: Pro-inflammatory cytokines in control and newly diagnosed (pretreated and treated) chronic lymphocytic leukemia patients: A follow-up study

Parameters	Control (n=19)	Newly diagnosed CLL patients	
		Untreated (n=18)	Treated (4.5 months), n=14
IL-6 (pg/ml)	0.50±0.31 ^a	2.53±1.98 ^b	1.36±0.98 ^b
IL-10 (pg/ml)	48.31±22.89 ^a	614±301 ^b	101.4±49.3 ^c

Values are expressed as mean±SD. Values with different letters within each parameter are significantly different ($P < 0.05$). CLL=Chronic lymphocytic leukemia, SD=Standard deviation, IL=Interleukin

in compared with healthy control, ranged between (1.3 and 6.9 pg/ml). Various studies reported that IL-6 levels in the serum increase in CLL above the range in normal control subjects, however, leukemic cells do not appear to be the source of IL-6. Several investigators have suggested that IL-6 in CLL inhibits proliferation but prolongs survival (by suppressing apoptosis) of CLL cells.^[15,18]

The range of the IL-10 expression of the newly diagnosed CLL patients was between (212 pg/ml) (which is the lowest positive value) and (987 pg/ml) which is the highest positive value with a mean 614 ± 301 , with all the morphologically diagnosed CLL cases showed positive expression for IL-10. Various studies reported that IL-10 levels were increased in CLL and that were associated with poor prognosis.^[31,32] Several studies also revealed a significant elevation in IL-6 and IL-10 plasma levels of CLL patients in comparison to healthy control group.^[33,34] Such increase in the level of IL-10 might be due to its production by malignant cells or by different cells of the immune system, including T and B lymphocytes, macrophages and monocytes.^[35] After treatment with immunochemotherapy (RFC protocol) for a median of 4.5 months for the newly diagnosed CLL patients normalized only ZAP-70 levels, but not other parameters which may be due to combined effect of these drugs on lymphocytes.

Conclusion

ZAP-70 level was higher in CLL patients than control and immunochemotherapy can normalize this which indicated good response to treatment. On the other hand, IL-6 and IL-10 were also higher in patients with CLL but not affected by therapy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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