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Serum Interleukin 6 (IL -6) as Prognostic Marker in Egyptian CLL patients

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ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. Currently, several biomarkers are being used as CLL prognosticators, including elevated protein levels, elevated RNA levels, gene mutations, and epigenetic changes.

Materials and Methods: This study is a prospective study conducted on 55 patients newly diagnosed with CLL, serum IL-6 level was measured initially and after a 6-month treatment course. Correlation with the course of the disease and the known CLL prognostic parameters was done initially and after 6 months.

Results: The initial serum IL-6 level in the patient group (pre-treatment) ranges from 36-91 pg/mL (median 57), and in the patient group (post-treatment) ranges from 1-32 pg/mL (median 2). Serum IL-6 level was positively correlated with WBC count, β2 microglobulin, LDH, ESR, B symptoms, Uric Acid, BM Aspirate (% of lymphocytes), and Binet and Rai staging systems.

Conclusion: Serum IL-6 is a useful poor prognostic marker in newly diagnosed CLL patients; its prognostic value goes with the other known prognostic markers such as the BM lymphocyte count, ESR, and LDH.

Keywords: Chronic lymphocytic leukemia (CLL); IL-6; Prognostic markers; Cytogenetics

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults, accounting for approximately 30 % of all leukemia cases in European and North American countries[1].

The median age of patients at diagnosis is 70 years and only 10–15% of the patients are diagnosed at the age of 50 or less. It is characterized by accumulation of non-functional B-cells with a high expression of CD5, CD19, CD20 and CD23 and a low expression of surface Immunoglobulins IgM, IgD and CD79a compared to normal B-cells[2].

CLL develops through increased proliferation of immature lymphocytes in lymphoid organs, which results from an increased expression of antiapoptotic BCL-2 family proteins[3].

In case of an indolent form, the disease does not usually progress to a severe form, and the patient may survive for years without treatment, unlike aggressive forms, the cell number may quickly double and, as a result, the disease may be fatal within a relatively short period of time[4,5].

Several biomarkers are being used as CLL prognosticators, including elevated protein levels (e.g., TCL-1, ZAP-70, and CD38), elevated RNA levels

(e.g., CLLU1, LPL, and miRNAs), gene mutations (e.g.TP53, SF3B1, BIRC3, and NOTCH1) and epigenetic changes[6].

Since CLL patients usually bear several genetic abnormalities, a combination of various treatments is often used, and its choice should be tailored to the abnormalities present. For example, patients with del(17p) and/or a TP53 mutation are resistant to fludarabine, cyclophosphamide and rituximab, whereas alemtuzumab combined with methylprednisolone has been shown to be highly effective[7].

Cytokines are involved in every stage of cell life and death, and therefore play a key role in immune and inflammatory responses and the process of development, regeneration, and maintenance of homoeostasis in the body. The first identified cytokines were interleukins (IL) regulators of proliferation, differentiation and functioning of leukocytes and among that IL-6[8].

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, lymphocytes, and bone marrow stromal cells (BMSCs). Early reports implicated IL-6 as pro-tumorigenic factor in various human tumors[13].

IL-6 is also considered to be a key mediator of the body's defense responses, having a strong pleiotropic action. It exerts its effect on target cells by conjugating with a class I receptor, an IL-6Ra (CD126) subunit which binds ligands, and glycoprotein 130 (gp130), responsible for signal transduction[10].

MATERIALS AND METHODS

This study is a prospective case control study that was conducted on 105 subjects who were divided into case and control groups. The case group included 55 adult patients newly diagnosed with CLL, while the control group included 50 healthy subjects of matched age and sex. Patients with previously treated CLL and those with active immunological disorders were excluded from the study. The study was conducted in Clinical Hematology Unit in Ain-Shams University Hospital, Cairo, Egypt.

All procedures performed in our study were in accordance with the ethical standards of the ethical committee of Faculty of Medicine, Ain Shams University following the1975 Helsinki Declaration as revised in 1983. Informed consent was obtained from all participants included in the study.

Study procedures

- Serum IL-6 was measured in the patient group before and after 6 months of therapy, while in the control group it was measured once initially.
- All patients were started on chemotherapy according to Ain Shams University hospital protocols.
- Ten patients (18.1%) received oral chlorambucil 0.4 mg/kg biweekly, 35 patients (63.6%) received FC protocol every 28 days (Fludarabine 25 mg/m^2/day IV on days 1-3 plus Cyclophosphamide 250 mg/m^2/day IV on days 1-3), Ten patients (18.1%) received FCR protocol every 28 days (Fludarabine 25 mg/m^2/day IV on days 1-3 plus Cyclophosphamide 250 mg/m^2/day IV on days 1-3 plus Rituximab 375 mg/m^2 IV on day 1 of cycle 1 and 500 mg/m^2 IV on day 1 of cycles 2-6)

Sample collection and storage

- 2-3 ml of venous blood sample was withdrawn from each participant under complete aseptic conditions and was placed in a serum separator tube (SST) and allowed the samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Then we removed the serum and stored the samples at ≤ -20 °C.

Reagent preparation

- All reagents were brought to room temperature before use.

Wash Buffer

- 20 mL of Wash Buffer Concentrate was added to distilled water and 500 mL of Wash Buffer was prepared.

Substrate Solution

- Color Reagents A and B were mixed in equal volumes within 15 minutes of use while protected from light. 200 µL of the resultant mixture is required per well.

Human IL-6 Standard

The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

667 µL of Calibrator Diluent RD6F (for serum samples) was pipetted into the 100 pg/mL tube. 500 µL of the appropriate calibrator diluent was pipetted into each remaining tube.

The stock solution was used to produce a dilution series (below).

Each tube was thoroughly mixed before the next transfer.

The undiluted Human IL-6 Standard (300 pg/mL) serves as the high standard. The appropriate calibrator diluents serve as the zero standard (0 pg/mL).

Assay procedure

All reagents and samples were brought to room temperature before use. All standards, samples, and controls assayed in duplicate.

1. All reagents were prepared and working standardised as directed in the previous sections.
2. 100 µL of Assay Diluent RD1W were added to each well.
3. 100 µL of standard, sample, or control was added per well. Then Covered with the adhesive strip provided then incubated for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
4. Each one was aspirated well and washed; the process was repeated three times for a total of four washes. Then washed by filling each well with Wash Buffer (400 µL) using a squirt bottle. The liquid was completely removed at each step. After the last wash, any remaining Wash Buffer was removed by aspirating. Then the plate was inverted and blotted against clean paper towels.
5. 200 µL of Human IL-6 was added and Conjugated to each well. Then cover with a new adhesive strip then Incubated for 2 hours at room temperature.
6. The aspiration and the wash were repeated as in step 5.
7. 200 µL of Substrate Solution was added to each well then Incubated for 20 minutes at room temperature.
8. 50 µL of Stop Solution was added to each well. The color change in the wells was observed.
9. The optical density of each well was determined within 30 minutes, by using a micro-plate reader.

Calculation of results

The duplicate readings for each standard, control and sample were averaged and the average zero standard optical density (O.D.) was subtracted.

A standard curve was created by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve fit.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 22. Data were summarized using mean and standard deviation for quantitative data and using frequency (count) and relative frequency (percentage) for categorical data.

Comparisons between quantitative variables were done using the non-parametric Mann-Whitney tests and for categorical data the Chi square (c2) test was performed. Correlations between quantitative variables were done using Spearman correlation coefficient. P-values less than 0.05 were considered as statistically significant.

RESULTS

This study included 55 newly diagnosed adult CLL patients. The mean age of patients was 56.2 years (range, 44-72 years). The case group included 37 males (67.3%) while the control group included 32 males (64%).

According to the Rai staging system, 18% of our studied patients were stage I, 31% were stage II, 24% were stage III and 27% were stage IV.

The median WBC was 94 (range, 23-498 $\times 10^9$/L). The mean hemoglobin level was 11.55 (range, 6-16g/dl), mean platelet count was 223.7 (range, 57-420 $\times 10^9$/L). The mean β2 microglobulin was 5.45 (range, 3-9mg/L). The mean LDH level was 670.9 (range, 166-1856 IU/L). The mean 1st hour ESR was 99.3 (range, 2-160 mm). The mean uric acid level was 7.42 (range, 4-12 mg/dl) (Table1).

Table 1: Patients' initial laboratory results

Parameter		Result
WBC	Median (IQR)	94 (60 – 205)
	Range	23 – 498
Hemoglobin (HGB)	Mean±SD	11.55 ± 2.62
	Range	6 – 16
Platlets (PLT)	Mean±SD	223.71 ± 105.28
	Range	57 – 420
B2 Microglobulin	Mean±SD	5.45 ± 1.55
	Range	3 – 9
LDH	Mean±SD	670.91 ± 305.15
	Range	166 – 1856
ESR	Mean±SD	99.38 ± 40.61
	Range	2 – 160
Uric Acid	Mean±SD	7.42 ± 2.10
	Range	4 – 12
BM lymphocytes (%)	Mean±SD	85.78 ± 9.90
	Range	58 – 98

The initial serum IL-6 level in patient group (pretreatment) ranged from 36-91 pg/ml (median 57) versus 1-2 pg/ml in control group (median 1) with a highly significant difference (P value < 0.001). IL-6 level did not show a significant correlation with age and sex in both case and control groups.

The response to treatment was assessed following a 6-month chemotherapy course according to standardized criteria; where 78.2% of our studied patients achieved complete response, 20% achieved partial response, 1.8% had stable disease and none had a progressive disease.

Interleukin 6 level was significantly lower in the patient group following treatment (median 2 pg/ml) compared to the pretreatment level (median 57 pg/ml).

Known CLL prognostic markers as WBC count, LDH, B2 microglobulin and CD38 percentage were inversely correlated with the response to treatment (P value of 0.011, <0.001, 0.014, <0.001 respectively), while 17p deletion by FISH and the initial staging by Binet and Rai scores didn't show a significant correlation (P values 0.775, 0.135 and 0.058 respectively).

Pretreatment IL-6 level showed a highly significant correlation with WBC count, Uric acid level, LDH and B2 microglobulin (P value <0.001 for all parameters) and the percentage of BM lymphocytes (P value 0.002), but it didn't show a significant correlation with age, sex and ESR.

IL-6 level showed a highly significant correlation with other CLL prognostic markers as Binet and Rai staging at diagnosis and CD38 percentage (P value <0.001 for all parameters), also had a significant correlation with the B symptoms (P value 0.010), while correlation with 17p deletion by FISH was non-significant (P value 0.067).

The best cut off point for serum level of IL-6 (before treatment) in patients with CLL was found > 2 with sensitivity of 100%, specificity of 100% and AUC of 1.000 (Table 2).

The best cut off point for serum level of IL-6 (post treatment) in patients with CLL was found > 1 with sensitivity of 58.18%, specificity of 86% and AUC of 0.739.

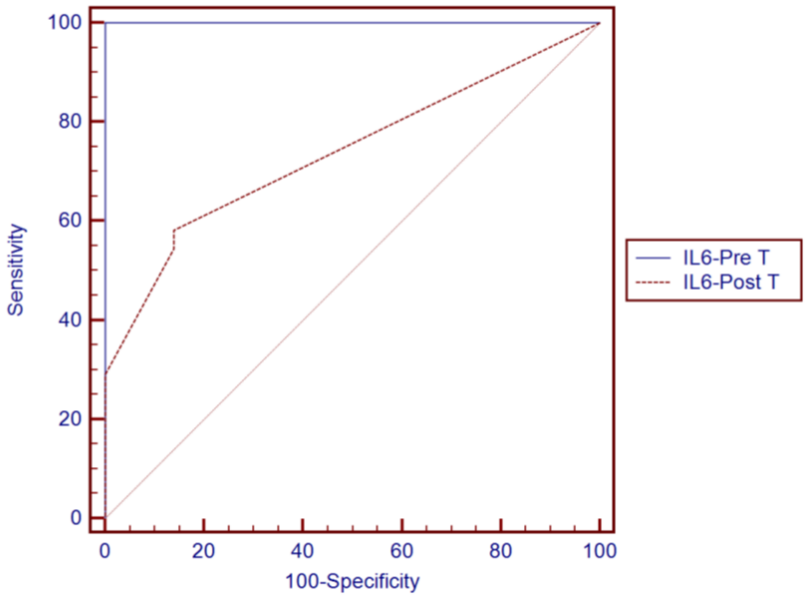

Figure 1. Correlation between response to treatment and IL-6 level

Table 2: Correlation between response to treatment and IL-6 level

Variables	Cut off point	AUC	Sensitivity	Specificity	+PV	-PV
Pre-treatment IL6	>2	1.000	100.00	100.00	100.0	100.0
Post-treatment IL6	>1	0.739	58.18	86.00	82.1	65.2

DISCUSSION

The results obtained from this study showed that the mean level of IL-6 was significantly higher in the patient group than in control group.

In the presented study we found that the mean pre-treatment level of IL-6 was higher compared to the post-treatment level.

Our finding goes with that reported by Ennas et al.[11], who found that Serum IL-6 levels were higher in CLL patients (median, 1.45 pg/mL; range, undetectable to 110 pg/mL) than in control subjects (median, undetectable; range, undetectable to 4.30 pg/mL) (*P* < .0001).

On the other hand, Antosz et al.[9] found that IL-6 and IL-10 expressions are statistically lower in B-CLL in comparison with the control group, in all cases (p<0.0001). This contradiction might be explained by that the study used PCR technique in measuring IL-6 Level not ELISA technique as we did.

In the current study we found that the level of IL-6 was higher in the patient group before treatment than in the same group after 6 months of treatment. Going with our results, the study done by Robak et al.[12] found that in the patients - post treatment - the IL-6 serum level was significantly lower than in the same patients before treatment (P<0.02).

Our results showed that after 6 months of treatment IL-6 median level was the lowest in patient who achieved CR (complete response) compared to patient who achieved PR (partial response) and was highest in non-responders.

Robak et al.[12] also confirmed our results as they stated that the highest concentration of IL-6 was found in patient with NR and the lowest

concentration was found in the patients with complete remission (CR; median 1.4pg/ml; P<0.02).
We have also shown that IL-6 was at its lowest concentration in patients with stage A Binet Staging system, median in stage B Binet Staging system, and at its highest level in stage C Binet Staging system patients.
In a similar study by Georgia et al.[13], they found that IL-6 serum level was correlated with Binet stage (P = 0.0433) being higher in stage C, followed by stage A, whereas stage B cases displayed the lower levels of IL-6.
On the other hand, Garley et al.[14] inferred that patients with B-cell chronic lymphocytic leukemia did not show any statistically significant differences in the expression of IL-6 at various stages of the disease's advancement.
Our explanation about these controversies maybe due to the sample size – as Garley et al. selected only 23 patients – and they used Western blot technique to measure IL-6 level.
Our study showed that IL-6 levels were going hand in hand with lymphocytic count as IL-6 levels being higher in patients with high lymphocytic count. This result was supported by Robak et al.[12] who found a significant positive correlation between levels of IL-6 and lymphocytes count in CLL patients (p=0.423; P<0.001).
In addition, we also found that IL-6 level was higher in patients with 17P deletion than patients with negative 17P deletion. Moreover, we found higher IL-6 level in positive CD 38 patients than negative CD 38 patients.
Robak et al.[12] supported our results as they found that the concentration of IL6 was higher in patient with 17P deletion than patients with negative 17P deletion and higher in positive CD 38 patients compared to CD38 negative patients.

CONCLUSION

Serum IL6 level was elevated in CLL patients at the time of presentation and decreased after treatment. Serum IL-6 level correlates with CLL prognosis and matches with the other known poor prognostic parameters in CLL patients.

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