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Evaluation of CD69 Expression and Plasma Interleukin - 6 in Chronic Lymphocytic Leukemia

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Abstract:

Chronic lymphocytic leukaemia (CLL) is due to the accumulation in bone marrow, peripheral blood, and lymphoid tissues of monoclonal B lymphocytes with a distinct immunophenotype, CD69 is an integral membrane protein belonging to the lectin family. It is expressed after activation in all bone marrow-derived cells. CLL exhibits features of activated and antigen-experienced B lymphocytes and CD69 overexpression, IL-6 may promote disease progression either directly through effects on cell survival and proliferation or indirectly through effects on the microenvironment.

Objective:

Evaluate the expression of CD69 in newly diagnosed patients with CLL by flow cytometry, To assess the plasma level of IL6 in those patient**,** To find the correlation of these markers with clinical features and hematological parameters.

Patients, materials and methods: across- sectional was conducted on Thirty adult patients with newly diagnosed CLL were consequently selected from the Hematology outpatient clinic at the Medical City, Oncology Teaching Hospital during the period from August 1, 2016 to December 30, 2016. a 5ml venous blood were obtained and collected in two K3-EDTA tubes from the patients for detection of CD69, CD38 by flow-cytometry and IL6 enzyme-linked immuno- sorbent assay and 1ml of blood was collected in plain tube for LDH estimation by kinetic assay method.

Results: The mean age group of CLL patients included in this study was 62.4 ± 12.2 (mean ±SD) years with M: F ratio of 2:1. CLL patients studied were: 14 patients (46.7%) were in the high risk group ,10 patients (33.3%) were in the intermediate risk group and 6 patients (20%) were in the low risk group. CD69 expression was detected in 46.7% ,14 /30 patients and 12 of them in high risk group. The median plasma IL-6 was 49.7 ng/L in CLL patients and 42.1ng/L in control, IL-6 was significantly higher in patients compared to controls

Conclusion: The current study showed that the expression of CD69

INTRODUCTION:

CLL is the most common type of adult leukemia in the western world. It is a malignancy of mature B cells involving blood, bone marrow (BM), and lymphoid tissues, and its cells arise from polyclonal expansion of CD5+ B lymphocytes transformed into a monoclonal population by mutational agents [(1)]. Two major clinical staging systems (Rai and Binet staging), mainly based on tumor load, were developed to estimate prognosis in CLL Binet and Ria staging system CLL has different prognosis, it may be growing slowly with minor changes in blood cells count; or may be presented as rapid growing malignancy. [(2)]

Diagnostic criteria [(3)]

According to the International Workshop on CLL (IWCLL), the diagnosis of CLL is sustained by the following parameters:

1. Presence in peripheral blood of>5×109/L monoclonal B lymphocytes persisting for at least 3 months.
2. Demonstration of the clonality of the population (κ/λ analysis).
3. Characteristic immunophenotype:

SmIg weak, CD5+, CD19+, CD20weak, CD23+, FMC7 (a CD20 epitope) and CD79b are usually absent or weakly expressed.

Many studies concentrated on the identification of surrogate markers with prognostic value, and whose expression could be easily verifying by flowcytometry; for example, expression of CD38, expression of immunoglobulin M (IgM), and IgD heavy chain in CLL have been shown to correlate with unfavorable prognosis. [(2)]

As CLL is not associated with recurrent balanced chromosomal translocations. For this reason, several biological parameters have been added to the staging systems to differentiate prognostic subsets [(4)].

CD69 is an integral membrane protein belonging to the lectin family. It is expressed after activation in all BM-derived cells except erythrocytes [(5)]. CLL exhibits features of activated and antigen-experienced B lymphocytes and CD69 overexpression [(6)]. High expression of CD69 in CLL cells has been shown to be associated with a more advanced stage and decreased survival. An association has also been demonstrated between mutation status and CD69 expression. [(7)]

Cytokines are intercellular short-acting soluble mediators that are involved in the pathogenesis of cancer. Cytokines can either be produced by or exert effects on neoplastic or reactive cells, cytokines may be utilized as a marker of immunity status and/or prognosis in cancer.

Interleukin-6 is a multifunctional cytokine involved in the regulation of various cellular functions, among them proliferation, apoptosis, angiogenesis, differentiation and regulation of immune response, being also implicated in the pathogenesis of several lymphoproliferative disorders suggested an important role of IL-6 in survival of CLL cells [(8)]. CLL patients with higher IL-6 levels tend to have more aggressive diseases [(9)].

PATIENTS, MATERIALS, AND METHODS

prospective cross-sectional study was conducted on thirty patients with new diagnosis of B-CLL, the study was conducted at the Hematology outpatient clinic at

Oncology Teaching Hospital of the Medical City in the period from August 1, 2016 to December 30, 2016.

Diagnosis was based on morphology and immunophenotyping of the PB samples by an expert hematopathologist in the Teaching Laboratories and Nursing Home Hospital/ flow cytometry department of the Medical City in Baghdad. A written informed consent obtained from all patients before study.

The panel that was used for diagnosis of CLL was SmIg, CD5, CD23, FMC-7, and CD79b that typically show score (4–5) according the FCM scoring system for diagnosis CLL. [2]

FC for the prognostic markers CD 38 and CD69 were done in flow cytometry department of the Medical City in Baghdad.

Inclusion Criteria

1. The patients were adult collected regardless to gender, age and the stage of the disease.
2. All CLL patients were newly diagnosed, and they were not receiving any chemotherapy before the time of collecting blood samples.

Control Group

The control group included 30 age- and gender-matched healthy volunteers, based on their CBC and C-reactive protein, were used for comparison with the patient's study groups for IL-6 assay.

Blood sampling

From each patient included in this study, a 5ml venous blood sample by venipuncture from antecubital fossa was obtained under aseptic technique and the samples were collected as 2ml in K3-EDTA tubes for CBC.

Two mls in K3-EDTA tubes for IL 6 assay to separate plasma after centrifuged for 10 minutes at 3000 r.p.m and then collect 400µl from the supernatants which were carefully stored at -40℃ until the assay performed.

One ml of blood was added to plain tube, after clotting at room temperature, serum obtained by centrifugation for 10 minutes at 3000 r.p.m used for LDH estimation.

From controls 3 ml of blood collected as 2ml in K3-EDTA tube (for CBC and IL 6 assay) and 1ml in plain tube for C- reactive protein.

CBC including Hb, WBC count and differential, platelet count using automated haematology analyser (Cell-DYN, RUBY Abbott Diagnostic, USA).

Reticulocyte count was done manually and stained with reticulocyte stain (New methylene blue stain) using the standard procedures [10] along with direct antiglobulin **(**Coombs) test by a spin tube technique [11] to exclude hemolytic anemia for the purpose of clinical staging.

Flow cytometric Immunophenotyping[12]

Four-color immunophenotyping data were attained using a FACSCalibur flow cytometer (Partec Cyflow ® Cube 6, Germany). Fluorochrome-conjugated monoclonal antibodies that usually used as follows: CD3; CD5; CD10; CD19; CD20; CD23; CD45 and FMC-7; CD38, and IgM, and IgD. List mode files were analyzed using Cell Quest software (BD Biosciences). Atypical immunophenotypic characteristics in B-CLL were bright CD20 fluorescence, bright surface Ig light chain fluorescence, or positivity for FMC-7, and the positivity for CD38 was determined by comparison with the isotype control, and the CD38 + percentage cells among gated CD19+/CD5 + cells were calculated. The antigen expression (CD38) was considered to be positive when the percentage of positive cells was equal or >7%([13) (14) (15)

CD69 antigen expression was considered to be positive when the percentage of positive cells was equal or greater than 30 %. [16) (17)

Plasma IL6 assay.

Laboratory kit: Human Interleukin 6 Enzyme Linked Immunosorbent Assay (ELISA) kits was used for quantitative determination of IL6 based on Shanghai Korian Biological Technology (China) ,Cat NO :E0090HU.

The assay was performed by (ELISA) technique at Immunology Department/Teaching Laboratories.

Principle of The test: The LDH method uses as a substrate L-lactate buffered at a PH of (9.4). Lactate dehydrogenase oxidizes the substrate in the presence of NAD^+ to yield pyruvate and NADH which absorbs at 340nm.

Lactate dehydrogenase activity concentration is measured as a rate reaction at 340/700 nm, proportion to the amount of lactate dehydrogenase in the sample.

The statistical analysis of this cross-sectional study performed with the IBM SPSS Statistics for Windows, SPSS 20.0.0, Minitab 17.1.0 software package and Microsoft Excel program Ver. 3. 2013, Chicago. While, categorical data described as count, percentages, and Chi-square test used to estimate the association between variables. Mann Whitney U test used to analyze the differences in median of two groups (if they do not follow normal distribution). Binary logistic regression analysis used to calculate the odd ratio (OR) and their 95% confidence intervals, when the outcome can be categorized into 2 binary levels. Linear regression analysis performed to assess the relationship between different variables, r (correlation coefficient or standardized beta is a representative of magnitude and direction of the relationship), r<0.25 weak, 0.25 – 0.5 mild, 0.5 – 0.75 moderate, >0.75 strong correlation. Negative sign indicates inverse relationship but positive sign represents direct relationship.

For the tables with frequencies, range, mean, and standard deviation values were considered statistically significant difference when $P < 0.05$.

RESULTS

Thirty adult newly diagnosed CLL patients were enrolled in this study; the mean age was 62.4 ± 12.2(mean± SD) years with a range of 30-85 years. Highest percentage of patients (53.3%) fall within the age group (60-69) years old. Chronic lymphocytic leukemia cases were observed more in males 20/30 (66.7 %) than in females 10/30 (33.3 %) with an M: F ratio of 2:1. The common clinical presentation of CLL patients included in this study were lymphadenopathy and splenomegaly (36.70% and 26.70%, respectively), followed by pallor, fatigue.

By applying the Modified Rai staging System 14 patients (46.7%) were in the high risk group, 10 patients (33.3%) were in the intermediate risk group and 6 patients (20%) were in the low risk group. The median of absolute lymphocyte count (ALC) was 74.1 $\times 10^9$/L, while the median of Hb concentration was 11.9 g/dl and the median of platelet count was 157.5×10^9/L as shown in table 1.

CD Markers Expression:
CD69 expression was detected in 14/30 patients (46.7%) while CD38 expression was detected in 15/30 patients (50 %). The median of positive expression of CD69 was 46.8% with a range 42.7%-50.6% while for CD38 was 12.2% with a range 10%-20%. (Table 2).

IL6 in CLL patients
The median plasma IL-6 was 49.7 ng⁄L in CLL patients and 42.1 ng⁄L in control. IL-6 was significantly higher in patients compared to control as illustrated in table 3

LDH IU/L median level in CLL patients were 480 (230.8 –509.3) with range of (151 – 889).

Relationship of biological markers expression to modified RAI staging system.
IL-6, CD69, CD38 and LDH all had significantly higher levels in high RAI stage compared to low and intermediate groups as illustrated in table (4).

Correlation between CD69 and different variables.
There was direct and significant correlation between CD69 with Modified RAI, WBC, ALC, CD38, LDH, splenomegaly and hepatomegaly, but it was inverse and significant between CD69 and, platelet count as illustrated in table 5
Comparison between group I CLL patient's n =16 [CD69<30] and group II CLL patients [CD69≥30] n= 14 as regards some clinical and laboratory parameters as shown in table 6

Correlation between IL-6 and different variables:
There was direct and significant correlation between IL-6 with Modified Rai, WBC, ALC, CD38 and LDH. And it was inverse between IL-6 and hemoglobin as illustrate in table 7.
Comparison between CD 69 and IL-6 the median of IL6 in group II is significantly higher than group I.

Validity of CD69, CD38 and IL6 expression as prognosis predictors in relation to Modified Rai staging.
As shown in Table (8,9); IL-6 offer the best prediction of poor prognosis according to Modified RAI classification followed by CD38 and CD69 in this research

Table 1. The hematological parameters of CLL patients (n=30).

Parameters	Median	Inter-quartile range (IQR)	Range
Wbc count (x 10^9/L)	85.1	28.6-160.3	11.5-194
ALC (x 10^9/L)	74.1	21.9 – 122.3	5.5-168
Hb (g/dl)	11.9	10 – 13.2	6-15.3
Platelets (x 10^9/L)	157.5	119.8 – 241.5	37-324

Table 2 Median and range of the CD69&CD38 markers expression of the patients, n=30.

Markers		Median% (IQR)	Range%
***CD69**	Positive	46.8 (42.7 – 50.6)	(35 - 60)
	Negative	5.0 (2.8 – 15.8)	(0.22 -19.77)
****CD38**	Positive	12.2 (10 – 20)	(7.5-24)
	Negative	0.4 (0.1 – 1.0)	(0 - 3)

*CD69 considered positive ≥30%, ** CD38 considered positive ≥7%.

Table 3: comparison of median IL-6(ng⁄L) level between CLL patients and control

	Patients	Control	P value
Median (IQR)	49.7 (40.3 – 86.1)	42.1 (33.6 – 46.9)	0.002 [Sig.]
Range	15 – 359	12.8 – 57.91	

Table4: comparison between biological markers and modified RAI staging system.

		Low	Intermediate	High	P value
CD69	<30%	6 (100.0%)	8 (80.0%)	2 (14.3%)	<0.001
	≥30%	0 (0.0%)	2 (20.0%)	12 (85.7%)	
CD38	<7%	6 (100.0%)	8 (80.0%)	1 (7.1%)	<0.001
	≥7%	0 (0.0%)	2 (20.0%)	13 (92.9%)	
IL-6(ng/L)	Median IQR	35.6 (27.9 – 41.2)	46.5 (34.6 – 48.9)	86.8 (71.6100.4)	<0.001
LDH(IU/L)	Median IQR	209 (180 – 341)	300 (193 – 425)	500 (496 – 602)	<0.001

Table 5: correlation between CD69 and different variables		
Variables	**r**	**P value**
Age	0.274	0.143
Modified RAI	0.672	<0.001 [Sig.]
WBC count (x 10^9/L)	0.433	0.017 [Sig.]
ALC (x 10^9/L)	0.497	0.005 [Sig.]
Hb(g/dl)	-0.605	<0.001 [Sig.]
Platelet count (x 10^9/L)	-0.406	0.006 [Sig.]
CD38	0.879	<0.001 [Sig.]
LDH(IU/L)	0.772	<0.001 [Sig.]
	OR	P value
Gender	0.912	0.088
Splenomegaly	1.579	0.024 [Sig.]
Hepatomegaly	1.350	0.005 [Sig.]
Lymphadenopathy	0.955	0.353

Table 6 Comparison between group I and group II patients as regards some clinical and laboratory parameters.				
Parameter		**Group 1(n=16) (CD69<30)**	**Group 2 (n=14) (CD69≥30)**	**P value**
Age	Median IQR	63.00 (55 -70)	65.00 (63 -70)	0.313
ALC (x 10^9/L)	Median IQR	34.40 (18.10-86.40)	115.00 (23.20-155)	0.045 [sig.]
Hb(g/dl)	Median IQR	12.20 (11.50-14.50)	10.00 (7.83-12.80)	0.005 [sig.]
Platelet count (x 10^9/L)	Median IQR	188 (153-253)	132 (86-165)	0.043 [sig.]
LDH (IU/L)	Median IQR	235 (190 -366)	500 (498 -607)	<0.001 [sig.]
Gender	Female	3	7	0.070 [sig.]
	Male	13	7	
Modified Rai staging	Low	6	0	<0.001 [sig.]
	Intermediate	8	2	
	High	2	12	
CD38	<7%	15	0	<0.001 [sig.]
	≥7%	1	14	
Spleen	No	11	0	<0.001 [sig.]
	Yes	5	14	
Liver	No	16	4	<0.001 [sig.]
	Yes	0	10	
Lymph node	No	6	7	<0.001 [sig.]
	Yes	10	7	

Table 7 correlation between IL-6 and different variables		
Variables	**r**	**P value**
Age	0.170	0.194
Modified RAI	0.453	0.012 [Sig.]
WBC count (x 10^9/L)	0.387	0.035 [Sig.]
ALC (x 10^9/L)	0.452	0.012 [Sig.]
Hb(g/dl)	-0.505	0.004 [Sig.]
Platelet count (x 10^9/L)	-0.141	0.456
CD38	0.674	<0.001 [Sig.]
LDH(IU/L)	0.541	0.002 [Sig.]
	OR	P value
Gender	0.991	0.234
Splenomegaly	1.059	0.053
Hepatomegaly	1.009	0.275
Lymphadenopathy	0.994	0.404

Table 8 : receiver operator characteristics of CD69, CD38 and IL-6 poor prognosis			
	AUC	Optimal cut point	P value
CD69	**0.893**	19.77	<0.0001
CD38	**0.911**	3	<0.0001
IL-6	0.999	50.08	<0.0001

Table 9: Values of validity of CD69, CD38 and IL6 as prognosis predictors in comparison to Modified Rai stage of the patients, n=30.						
	Cut point	Sensitivity	specificity	Accuracy	PPV*	NPV**
CD69	**≥19.77**	85.7	87.5	86.7%	85.7	87.5
CD38	**≥3.0**	92.9	87.5	90.0%	86.7	93.3
IL-6	≥50.08	99%	99%	99%	99%	99%

DISCUSSION

CLL is a low-grade B-lineage lymphoid malignancy characterized by absolute lymphocytosis in the peripheral blood and bone marrow at the time of diagnosis. [18]

In this current study the mean age of the patients included was 62.4+12.2 SD years, and the range of the age was between 30-85 years old, which was close to the results obtained by other Iraqi studies [19-20] and other studies in Asian countries [21]. The most common presenting clinical feature of CLL in this study was lymphadenopathy followed by splenomegaly, and only 6.7% of the patients was asymptomatic at diagnosis. Those results were comparable with other Iraqi studies. [19,22,23] and similar finding regarding the common presentation in many Western studies but proximately 50% of patients with CLL were asymptomatic at diagnosis [24].

The low incidence of asymptomatic patient in Iraq may be attributed to late diagnosis due to lack of routine checkup in Iraqi patients. Thus, they will be presented with advanced clinical stage.

For the hematological parameters of the patients, the median of Hb concentration was 11.9 g/dl with a range (6-15.3g/dl), which was close to the results obtained by other Iraqi studies. [20,25]

By applying the Modified Rai staging system on our patients, 46.7% were in high-risk stage, 33.3% in the intermediate stage and only 20% were in the low-risk stage. Those results were comparable with other Iraqi studies [19,23,26,27,28]. However, Western countries studies showed that 40 -60% of all patients with CLL were in the early clinical stage of the disease at the time of diagnosis [29].

Regarding the CD markers expression, among 30 newly diagnosed CLL cases that were included in this study CD38 was expressed in 50% of CLL cases (15 out of 30 cases). Similar result was reported by other Iraqi study Thabit ZA et al. 2015 [22] but the result was higher than that reported by Hassanein NM et al. 2010 (32.4%) [30] and Hus I et al. 2006 (33.3%). [31] These differences may be due to the choice of an optimal cut-off for the number of CD38+ve cells as these studies used a 20-30% cut-off in contrast to this study which uses a 7% cutoff for CD38 expression. The largest studies to date found that a cutoff of 7% was best at separating different prognostic groups. [14,15]

For CD38, 13 outof 15 cases with CD38+ve were fall in High risk Modified Rai staging system, similar result of significant relation with advance stage was reported by D'Arena G et al. 2007, [3] Schroers R et al. 2005 [32], and Del Principe ML et al. 2006.[33]

CD69 was expressed in 46.7% of CLL cases (14 out of 30 cases), close to the result of Abd El-hadia E et al. 2015[16] and D Arena G et al 2001[34] who reported that CD69 was expressed in 57.5% ,52% in CLL patients respectively. But this study result was lower than the 62.3% reported by Smilevska et al. 2006 [35], may be attributed to lower cut off value for positivity they used (20%).

There was a significant reduction in Hb level, platelet count and elevation in LDH concentration in group II patients in comparison with group I patients which was consistent with Abd El-hadia E et al 2015[16] .

More over a significant relation was found between the two groups according to modified RAI staging so that 12/14 with CD69+ve present in high risk group and other 2/14 in intermediate risk group and no patients in low risk group Which was similar to the reports of other studies [16,34 ,17,36-38].

There was a significant association between the incidence of splenomegaly, hepatomegaly and lymphadenopathy among patients with high CD69 expression group II. This was partly in concordance with the results of Del Poeta *et al.* (2010) [36], who found that CD69 over expression was significantly related with splenomegaly and Abd El-hadia E et al 2015[16], who found that CD69 over expression was significantly correlated with splenomegaly and hepatomegaly.

A significant association between CD69 expression and CD38 was obtained. where in patients with high CD69 expression had significantly higher CD38 expression. This finding was confirmed by Elbaiomy M et al. 2016[37], Abd El-hadia E et al .2015[16] and Del Poeta G *et al.* (2010) [36] (2012) [17], the latter was reported that CD69 expression was significantly correlated with CD38, CD49d, and ZAP-70 and immunoglobulin variable heavy chain (IgVH) mutational status; they also demonstrated in a large series of CLL patients that CD69 protein expression was an independent risk factor for PFS and OS.

The rapidly up –regulated CD69 when cellular activation started ,may be responsible for the transduction of BCR–mediated signals in a better way with the co-expression of CD38 that has an important role to tranduce BCR-

mediated signal .[39] Such increased intracellular signaling may reflect ongoing stimulation ,thereby influence the proliferation or survival of CLL cells leading to a tendency toward disease progression and advanced stages and hence explaining the more aggressive disease course observed in these patients .[17]

Regarding LDH; had significantly higher levels in high RAI stage compared to low and intermediate groups and this result was consistent with that obtained by Shen QD et al .2007[40] and Mani R et al. 2006[41] who stated that increased serum LDH was commonly interpreted as reflecting high tumor burden or tumor aggressiveness and high serum LDH carries a poor prognosis in CLL.

For IL-6 this study has shown that plasma level of IL‑6 was significantly elevated in newly diagnosed CLL patients compared to controls and this result was consistent with that obtained by Iraqi studies. [42,43]

We found that IL-6 plasma levels elevated significantly in patients with CLL with advanced stage of the disease (Rai stage III /IV) which was supported by other studies. [44,45-46,47]

high IL-6 levels directly correlated with serum LDH level. Similar result has been reported with Fayed L et al 2001. [44]

IL-6 is inversely correlated with Hb level; this was in concordance with the other studies [153,155]. Increasing levels of IL-6 significantly correlated with white cell count and no significant correlation was found with platelet counts supported by Lai R et al .2002. [45]

In our study there was significant correlation between IL6 and CD38, Lai R et al .2002 stated that IL-6 was a predictor of survival in CD38-positive CLL patients and may be able to improve significantly the prognostic value of CD38 positivity in these patients. [45]

In conclusion, we found that IL-6 plasma levels was elevated significantly in patients with advanced stage of the disease and that IL-6 was an important prognostic factor in these patients.

Regarding the assessment of the validity of CD 69, CD38 and IL6 as poor prognosticators of CLL cases in relation to modified Rai staging, we found that IL6 is highly sensitive and specific with the highest accuracy (99%) than others, but unfortunately, no other studies were found for comparison.

CONCLUSIONS:

1.The expression of CD69 on CLL was detected in about half of patients.

2.The adverse prognostic impact of CD69 was consistent with the demonstration of higher expression levels of CD69 in CLL cells in advanced-stage patients according to Modified Rai staging system.

3.Plasma level of IL-6 was significantly elevated in newly diagnosed CLL patients compared to controls and elevated significantly in patients with CLL with advanced stage of the disease so it was important prognostic marker.

4.In this research; IL-6 offer the best prediction of poor prognosis with 99% sensitivity, specificity and accuracy.

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