Original article

Flowcytometric Measurement of CD5, CD23, and CD38 expression as a diagnostic and prognostic markers in CLL patients

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ABSTRACT

Background: B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal malignancy characterized by an accumulation of terminally differentiated small and anergic B lymphocytes in the blood, bone marrow and other tissues. CLL is typically characterized by CD5+, CD23+, CD22 -, CD79b-, with weak expression of surface Ig. CD5 also is expressed in B1 subset of human B –lymphocytes. Mature B cell malignancies, such as B-cell chronic lymphocytic leukemia, are mostly CD5 +. CD23 promotes the activation and proliferation of normal B lymphocytes and has an important role in the process of malignant transformation in B-CLL.CD38 is expressed on the surface of leukemic cells in a significant percentage of patients with B-cell chronic lymphocytic leukemia (B-CLL).Its expression has prognostic value in CLL. The current immunophynotype antigens is used to diagnosed as CLL cases, and by using the modern multicolor Flow Cytometry, which made it possible to determine the expression of several such antigens on specific cell populations of the CLL cases.

Objectives: To measure the expression of CD5, CD23, and CD38 antigens on the B-cells of morphologically diagnosed CLL cases, and showing their correlation with the hematological parameters, and with each other.

Material and methods: A prospective cohort study including 20 patients including 11 females and 9 males morphologically diagnosed with CLL. The patients were attending the National Center of Hematology, Al-Yarmouk teaching hospital for the period from November 2012 to March 2013. A total of 2 ml of venous blood were collected from all patients who were selected randomly with respect to age, sex, duration and stage of the disease. The diagnosis was done by

measuring and calculating the total number of blood cells and lymphocytes and hemoglobin and other by using autoanalyzer blood counter, then flowcytometry was used to measure the appearance of antigens surface CD5 and CD23 CD38.

Results: The mean of age of all patients included was 61.95+8.88 SD, and a range of (45-75) years old. There were 11 (55%) males patients, the most common symptoms of patients is an enlarged spleen (45%). 85% of patients who were in Binet stage C, the most advanced stage of the disease. Within Binet stage C there was 94.1% percent of patients showed moderate intensity expression of CD5 and CD23, while 64.7% of them for CD38. There was no statistical significance of CD5, CD23 in relationship to age, hemoglobin or platelet, while CD5 showed a significant relationship with lymphocytes count and the total number of white blood cells (P <0.05).CD38 showed significant relationship with hemoglobin (P <0.05). There is a significant correlation between the CD5 and CD23 P) <0.05), while the CD38 show positive correlation with CD23) P <0.05.

Conclusions

1-There is a significant correlation between CD5 expression and absolute lymphocyte count, so higher peripheral blood lymphocyte associated with greater CD5 antigen expression.

2-There is a significant correlation between CD5, and CD23 expression, so high CD5 expression associated with high CD23 expression.

3-There is a significant negative correlation between CD38 expression and Hb level that reflects a prognostic significance.

4-There is a correlation between CD38 expression and CD23 expression.

5-No correlation between the intensity of expression of CD5, CD23, and CD38 and stage of the disease.

6-No correlation between CD38 expression and age, WBC count, and lymphocyte count.

Keywords: Chronic Lymphocytic Leukemia, Flowcytometry, CD5, CD2, CD38

Introduction

B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal malignancy characterized by an accumulation of terminally differentiated small and anergic B lymphocytes in the blood, bone marrow and other tissues. These malignant cells can be identified by their varying surface membrane molecules, many of which are quite different to those expressed by normal cells and other lymphoproliferative disorders [(1)]. CLL is typically characterized by CD5+, CD23+, CD22 -, CD79b-, with weak expression of surface Ig [(2)]. CD5 is a T-cell marker of 65000-67000 Dalton that also is expressed in B1 subset of human B – lymphocytes. Mature B cell malignancies, such as B-cell chronic lymphocytic leukemia, are mostly CD5 +(3). CD23 antigen, a trans-membrane glycoprotein, promotes the activation and proliferation of normal B lymphocytes and has an important role in the process of malignant transformation in B-CLL [(1)]

CD38 is a transmembrane glycoprotein was initially characterized in 1980 as a T-cell differentiation antigen. In the following years, several studies showed that CD38 expression is not limited to T cells but is widely expressed on different hematopoietic and non-hematopoietic tissues. The strength of expression of CD38 on hematopoietic cells varies with the stage of maturation, the type of activation, and the milieu in which activation takes place. It expressed on the surface of leukemic cells in a significant percentage of patients with B-cell chronic lymphocytic leukemia (B-CLL).Its expression has prognostic value in CLL [(4)].

The current study involved measuring the expression of these markers on lymphocytes in twenty patients, who were morphologically diagnosed as CLL cases, by using the modern multicolor Flow Cytometry, which made it possible to determine the co expression of several such antigens on specific cell populations of the CLL cases [(5)].

Symptoms and signs of CLL

It is not unusual for a patient to feel entirely healthy with no symptoms whatsoever when a routine blood count reveals an absolute lymphocytosis requiring additional follow-up investigations that establish a diagnosis of CLL. On the other end of the spectrum is a patient who presents with all of the typical “B” symptoms of lymphoma (i.e., marked weakness, profuse night sweats, unintended weight loss, and fever without infection). Each of these extremes accounts for approximately 20% of cases at presentation. The remaining 60% have varying symptomatology with milder constitutional symptoms. Most patients consult a physician because they have noted painless swelling of

lymph nodes, often in the cervical area (but also at times in any other lymph node-bearing site), that spontaneously waxes and wanes but does not altogether disappear [6,7].

Diagnosis of CLL: To achieve this, it is essential to evaluate the blood count, blood smear, and the immune phenotype of the circulating lymphoid cells [8].

1. Blood: The diagnosis of CLL requires the presence of at least 5×10^9 B lymphocytes/L (5000/μL) in the peripheral blood. The clonality of the circulating B lymphocytes needs to be confirmed by flowcytometry. The leukemia cells found in the blood smear are characteristically small, mature looking lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. Smudge cells, found as cell debris, are other characteristic morphologic features found in CLL [8].

2. Immunophynotype: it is achieved by means of labelled antibodies that recognize specific epitopes of cellular antigens. In general, the most useful antibodies are monoclonal antibodies (McAb) produced by hybridoma technology but, for some antigens, antisera containing polyclonal antibodies (PcAb) are better. The technique employed for immunophenotyping is usually flowcytometry [9]. Table 1 shows the markers used for phenotyping CLL and table 2 shows the score in various B cell neoplasms.

FLOW CYTOMETRY

Immunofluorescence is the basis of flowcytometry immunophenotyping. Flow cytometry has the advantage over immunocytochemistry that it is rapid and quantification of the percentage of positive cells is more precise because many more cells are evaluated. On the one sample it is possible to determine forward light scatter (FSC) and sideways light scatter (SSC), examine the co expression of multiple antigens and quantitate the strength of antigen expression. The antibody is bound to a fluorochrome that absorbs light then emits light of a longer wave length, detectable at a specific relevant wave length[9].A stream of cells, labelled with an antibody conjugated to a fluorescent dye, flows past a detector so that cells can be counted and their FSC, SSC and fluorescence intensity can be characterized[9]. Co expression of antigens on single cells or populations of cells can be detected by using two or more antibodies conjugated to different fluorochromes with specific emission spectra[9]. Fluorescence intensity is determined by the fluorochrome used, the strength of binding and the number of epitopes carried on a cell. Immunophenotyping laboratories often use 'dim' and 'bright' to refer to fluorescence

intensity. As a broad approximation, signals between 0 and 101 can be regarded as negative, between 101 and 102 as weak(+), between 102 and 103 as moderate (++) and between 103 and 104 as strong (+++) [9]

Table(1) IMMUNOPHENOTYPE USED IN SCORING CLL(10).

Marker(result)	Score
SmIg(weak)	**1**
CD5(+)	**1**
CD23(+)	**1**
FMC7(- or weak)	**1**
CD79b(- or weak)	**1**
Total	**5**

Table (2)CLL Score in B-cell disorders[10].

Disease	Score
CLL	
Typical	**4-5**
Atypical;CLL/PL	**3-5**
B-prolymphocytic leukemia	**0-1**
Hairy cell leukemia	**0-1**
NHL with leukemia*	**0-2**

***Follicular lymphoma, MCL, SMZL**

as informative as the core biopsy regarding overall cellularity and degree of infiltration[10].Bone marrow aspirate smears reveal a lymphocytosis of ≥30% of all nucleated cells in the bone marrow differential count. Bone marrow biopsy reveals that the marrow invariably is infiltrated with leukemic lymphocytes. There are four patterns of marrow involvement. In approximately one-third of patients, the marrow has an interstitial, or lacy, pattern, which is associated with a better prognosis and/or early stage disease. Approximately 10 percent of patients present with a nodular pattern of marrow involvement, and approximately 25 percent have a mixed nodular-interstitial pattern. These patterns also are associated with a better prognosis. A quarter of the patients present with extensive marrow replacement, producing a diffuse pattern that is associated with advanced clinical stage and/or more aggressive disease[11]

Staging of CLL

1.The two widely accepted systems are those of Rai (1975) and Binet (1981)[10,12].

Rai staging system: This divides CLL into 5 stages: Rai stage 0: The blood lymphocyte count is too high, usually defined as over

10,000 lymphocytes/mm3 of blood. The lymph nodes, spleen, and liver are not enlarged and the red blood cell and platelet counts are near normal. Rai stage I: Lymphocytosis plus enlarged lymph nodes. The spleen and liver are not enlarged and the red blood cell and platelet counts are near normal. Rai stage II: Lymphocytosis plus an enlarged spleen (and possibly an enlarged liver), with or without enlarged lymph nodes. The red blood cell and platelet counts are near normal. Rai stage III: Lymphocytosis plus anemia (Hb less than 11g/dl), with or without enlarged lymph nodes, spleen, or liver. Platelet counts are near normal. Rai stage IV: Lymphocytosis plus thrombocytopenia (platelets count less than 100×10^9/L, with or without anemia, enlarged lymph nodes, spleen, or liver.

Binet staging system: The Binet classification integrates the number of nodal groups involved with the disease with bone marrow failure.

Binet stage A: Fewer than 3 areas of lymphoid tissue are enlarged, with no anemia (HB<10g/dl) or thrombocytopenia (platelets<$100x10^9$) [12].

Binet stage B: 3 or more areas of lymphoid tissue are enlarged, with no anemia or thrombocytopenia(12).

Binet stage C: Anemia and/or thrombocytopenia are present(12).

Materials and Methods

This study was conducted on twenty patients, including 11 males and 9 females, all with morphologically diagnosed CLL cases, 5 received treatment and 15 of them were newly diagnosed and no treatment was given. The patients were attending the National Center of Hematology, Al-Yarmouk teaching hospital, and some from private clinics and labs, through the period from November 2012 to March 2013.

Criteria for selection of patients:

1- All patients were diagnosed as CLL depending on morphology of their peripheral blood and bone marrow examination.

2-Randomly selected regarding the age, sex, duration and the stage of the disease.

3-Two cases were excluded as they showed negative expression for CD5, and CD23.

-Immunophenotyping by flowcytometry analysis to measure the expression of CD5,CD23, and CD38 surface markers was done for each patient at private lab.

Blood samples: A total of 2 ml of venous blood was collected by clear venipuncture into an EDTA tube, CBC was done for each sample by automated Abbot Ruby autoanalyzer at Al-Yarmouk teaching lab, blood film slides were revised for some of

the patients, and then the samples were sent within six hours to private lab for immunophenotyping.

Immunophenotyping for CD5, CD23 and CD38 expression were investigated by using four –color Cyflow® Cube 6 flow cytometry device (Partec Cyflow®, German), which is a fully equipped desktop Flow Cytometer (FCM). CyFlow Cube features a modular optical concept. This allows using different lasers as light sources (up to 2 light sources simultaneously: blue solid state laser: 488 nm and red diode laser: 638 nm and the detection of up to 6 optical Parameters (4 Colors + FSC + SS C) (parameters which denotes a measured property of the particles.) with selected PMTs with integrated electronic preamplifier for FSC, SSC, FL1-FL4(13). The CyFlow Cube allows easy optimization of the optics for any application by simple exchange of optical filters and (n) of cellsin a given volume (v), c = n/v. In the CyFlow® Cube 6, the volume is precisely measured directly by mechanical means, rather than indirectly with expensive and sometimes problematic beads, thus eliminating any errors related to varying bead concentrations or bead aggregations. The CyFlow® Cube 6allows the analysis of a fixed volume as defined by the distance between two platinum electrodes. The desired volume can also be freely selected, based on digital sample mirrors. Data acquisition, instrument control, and data analysis are controlled and performed by the CyView software. Compact flow cytometer for automated sequential analysis of single cells and microscopic particles (scatter particle size range: 50 nm -200µm), or cell subpopulation using True Volumetric Absolute Counting (TVAC). This advanced technology is solely based on the fundamental definition of absolute counting i.e.: the particle concentration (c) is equal to the counted number speed control by software [13]

Reagents: Product name: CyLyse Erythrocyte lysing reagent kit for wash and no wash procedures.

Contents: Reagent A for leukocyte fixation, 25 ml, Reagent B for erythrocyte lysing, 500 ml, Monoclonal Abs kits, Control beads

Description: CyLyse stands for an erythrocyte lysing reagent kit with a complete preservation of the surface proteins and practically no loss of cells.CyLyse is particularly suitable for absolute cell counting and for assays, demanding a minimum loss of leukocytes. Residual debris does not need to be removed by centrifugation due to the properties of the lysing reagent buffer. Fixative reagent A fixes and stabilizes the leukocytes. The fixed

samples can be stored for up to 24 hours at 2C-8C before analysis.

Method and Procedure [14] :1-Antibody labeling Antibody labelling was done by mixing 100 microliter of whole blood with conjugated antibodies (10 microliter) in a test tube, mixed thoroughly. Incubated for 15 minutes in the dark at room temperature.2-Leukocyte fixation For leukocyte fixation, 100 microliter of reagent A was added and mixed thoroughly and incubated for 10 min in the dark.3-Erythrocyte lysis For erythrocyte lysis 2.5ml of reagent B was added, shaken gently and incubated for 20min in the dark. Then the sample was analyzed on the flowcytometry. Some samples after fixation were stored at 2-8°C, protected from light, up to 24hr until analysis.

Flow cytometry data was 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 flowcytometry to provide an absolute value for the light intensity it measures is performed by comparing cell fluorescence with an external standard by using different commercially available beads in kits, which usually comprise two tubes. One tube contains four types of beads with four different levels of fluorescence uptake: one very dim, one very bright and two intermediate; the other tube contains blank (non-fluorescent beads) [15].

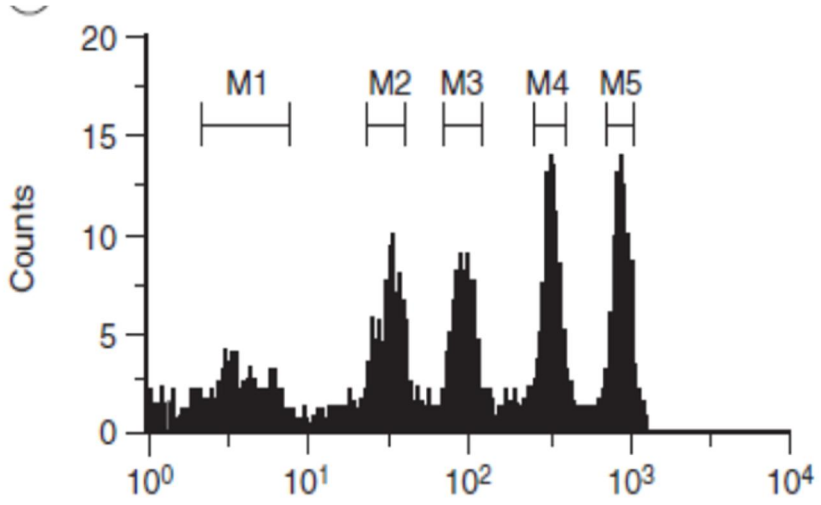


Fig (1) Beads fluorescence intensity showing five peaks: blank (M1); dim (M2); bright (M5); and intermediate (M3 and M4).

The instrument set up was so that the fluorescence signal of the tube with the blank (unlabeled) beads isolated in the region between 0 and 101 and four other peaks of fluorescence are seen along the axis of the relevant fluorochrome. The fluorescence voltage is established, and these settings maintained throughout the rest of the analysis of the unknown samples.(15).The samples for a particular McAb run with the fluorescence settings obtained from beads stained with the corresponding McAb, so that one fluorescence standard curve obtained for each McAb. The data obtained from the flow cytometer and, a standard curve is automatically produced. Identification of cells was performed using forward scatter (FSC) versus side scatter (SSC) parameters. Antigen expression was considered to be positive when the percentage of positive cells was equal or greater than 20%.[9]

Statistical analysis: Analysis of data was carried out using the available statistical package of SPSS-20 (Statistical Packages for Social Sciences- version 20). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). Pearson correlation was calculated for the correlation between two quantitative variables with its t-test for testing the significance of correlation. Statistical significance was considered whenever the P value was equal or less than 0.05.

Results

This study includes 20 adult patients with chronic lymphocytic leukemia, who were diagnosed morphologically by Lieshman stain on peripheral blood and bone marrow aspirates, biopsies stained by H&E.Immunophenotyping was done by Flow Cytometry to detect CD5,CD23 markers as diagnostic ,and CD38 as prognostic markers.

Clinical parameters

Age Groups: The mean of age of all patients included in this study was 61.95+8.88 SD, and a range of (45-75) years old. Table 3 shows that the highest percent of patients is within the age group of (60-69) years old.

Table (3) Age group distribution of the patients.

		No	%
Age (years)	<50	2	10.0
	50—59	4	20.0
	60—69	9	45.0
	=>70years	5	25.0
	Mean±SD(Range)	61.95±8.88	45-75

Gender: The patients included in this study were males (55%) and in females (45%). And within the most common age group of (60-69 yr), the percent of males (25%) was more than that of females (20%). As shown in table 4.

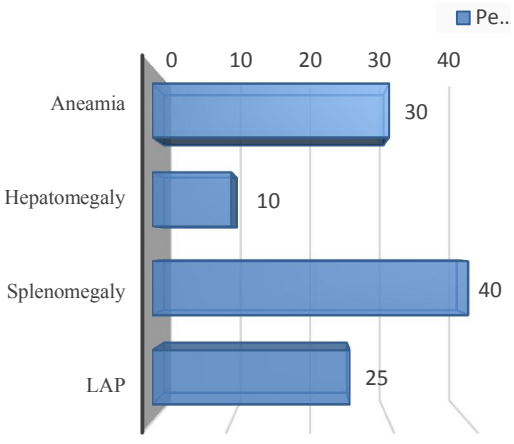
Table (4)The frequency of gender in relation to age group

Age group	Male	Female	Total
< 50	2 (10%)	0	2 (10%)
50-59	2 (10%)	2 (10%)	4 (20%)
60-69	5 (25%)	4 (20%)	9 (45%)
=>70	2 (10%)	3 (15%)	5 (25%)
Total	11 (55%)	9 (45%)	20(100%)

Clinical presentations

The clinical features of the patients are shown in the figure (2). The most common presenting feature of the patients is splenomegaly (45%) including (splenomegaly alone, splenomegaly with anemia or with hepatomegaly), followed by anemia (30%) as alone or with splenomegaly or with hepatosplenomegaly.

Fig. (2) presentation of the CLL patients.



Distribution of the patients according to Binet staging

The highest percent of the patients in this study (85%) fell within Binet stage C which is considered the most advanced stage in CLL patients, while (10%) stage B and (5%) stage A.

Hematological parameters

The mean Hb concentration was 10.98+ 2.84g/dl (mean+ SD) with a range between (5.6-16.5 g/dl)(16.5g/dl who was male 60 years old) and the Hb concentration was <10g/dl in 12 patients. The mean of lymphocyte count was 46.72+30.22x109/l (mean+SD), while the mean of platelets count was 162+71.28x109/l, and six presented with platelets count less than 100x109/l. The hematological parameters are depicted in table 5 below.

Table (5) Mean and Range of Hematological parameters of CLL cases

	Mean±SD	Range
WBC($x10^9$)	59.10±31.19	13.0-153.0
Lymphocyte($x10^9$/l)	46.72±30.22	7.5-141.0
Hemoglobin(g/dl)	10.98±2.84	5.6-16.5
Platelets($x10^9$/l)	162.0±71.28	30.0-280.0

Markers expression

The range of the percent of expression of the CD5 was between 20% which is the lowest positive value and 95% with a mean 65.65+22.6 (mean+SD), with all the morphologically diagnosed CLL cases show positive expression for CD5.

The percent of expression of CD23 range between 21%-96% (all show positive expression) with a mean of 67.08+21.41. While the CD38 range between 12- 86% with a mean of 38.79+22.58. Five patients out of twenty were negative (<20%) for CD38. Table 6 shows the expression of the three markers.

Table (6) Surface Markers CD5, CD23 & CD38 expression in CLL cases

Marker	Mean+SD	Range
CD5	65.65±22.60	20.0-95.0%
CD23	67.08±21.41	21.0-96.0%
CD38	38.79±22.58	12.0-86.0%

Hematological and clinical parameters in relation to Binet Staging: within Binet C stage, 12 (70.6%) out of 17 patients are with Hb less than 10g/dl .37.5% of patients are within (60-69) years old followed by 29.4% above70 years old. Within Stage C 52.9% are males while equal sex incidence within Binet stage B. 6% of patients had platelets less than $100x10^9$/l, six patients (35.3%) with splenomegaly, three (17.6 %) with lymphadenopathy and (5.9%) with hepatomegaly. As shown in table 7

Table (7) Hematological and clinical parameters in relation to Binet Staging

		A		B		C	
		No	%	No	%	No	%
Age (years)	<50	1	100	-	-	1	5.9
	50—59	-	-	-	-	4	23.5
	60—69	-	-	2	100	7	41.0
	=>70years	-	-	-	-	5	29.4
Gender	Male	1	100	1	50	9	52.9
	Female	-	-	1	50	8	47.1
Aneamia	Hb<10g/dl	-	-	-	-	12	70.6
	Hb≥10g/dl	1	100	2	100	5	29.4
Hepatomegaly	Yes	-	-	1	50	1	5.9
	No	1	100	1	50	16	94.1
Platelets	$<100x10^9$/l	-	-	-	-	6	35.3
	$\geq100x10^9$/l	1	100	2	100	11	64.7
Splenomegaly	Yes	-	-	2	100	6	35.3
	No	1	100	-	-	11	64.7
LAP	Yes	1	100	-	-	3	17.6
	No	-	-	2	100	14	82.4

Markers intensity in relation to Binet Staging: Within Binet Stage C;16(94.1%) of patients expressed moderate intensity for both CD5 and CD23, and one (5.9%) patient expressed mild intensity for those markers, while (11) 64.7% of them expressed moderate intensity for CD38, , and 5(29.4%) of patients showed negative expression for CD38 (<20%).Within Binet B: all patients expressed moderate intensity for CD5, CD38, while 50% showed mild intensity for CD23 and 50% moderate. This is illustrated in figure3.

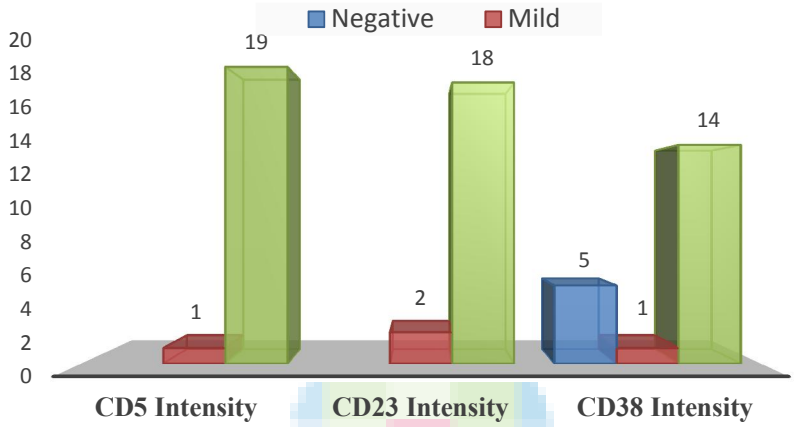


Figure (3) Intensity of markers expression in CLL patients

Markers expression in relation to Clinical and Hematological parameters

Within Binet group C patients , CD5,CD23 show no significant relation to each age, Hb or platelets, While CD5 show significant relation to lymphocyte counts (P <0.05) , and CD38 show relation with Hb level (P <0.05). As shown in table 8.

Table (8) Correlation between Clinical, hematological parameters and markers expression

C group patients (n=17)		CD5	CD23	CD38
Age (years)	r	0.995	0.641	0.361
	P	0.061	0.557	0.765
WBC ($X10^9$)	r	-0.996	-0.634	-0.353
	P	0.055	0.563	0.770
Lymphocyte	r	0.999	0.595	0.307
	P	0.024*	0.594	0.802
Haemoglobin (g/dl)	r	0.453	-0.480	-0.668
	P	0.701	0.681	0.047*
Platelets ($X10^9$)	r	0.727	-0.156	-0.464
	P	0.482	0.900	0.693

Markers Relation to each others

Within all Binets groups, there is a significant correlation between CD5, and CD23 ($P < 0.05$) , While CD38 show significant relation to CD23 ($P<0.05$) . This is expressed in table 9 below.

Table (9) Markers Relation of CLL patients

CD5		CD5	CD23	CD38
	R		0.544*	-0.037
	P		0.013*	0.878
CD23	R	0.544*		0.654
	P	0.013*		0.018*
CD38	R	-0.037	0.654	
	P	0.878	0.018*	
Pearson correlation with its t-test*Correlation is significant at the 0.05 level.				

Discussion:

B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes. It is the most common form of leukemia found in adults in Western countries[6]. These malignant cells can be identified by their varying surface membrane molecules, many of which are quite different to those expressed by normal cells and other lymphoproliferative diseases[1].

Clinical and Hematological parameters of the patients

In this study the mean age of the patients included was 61.95+8.88 SD, and the range of the age was between 45-75 years old, which are close to the results obtained by other Iraqi studies as shown in table (10)(16,17,18). These results also were comparable to the results reported by other studies in Asian countries [19]. While the results obtained from western countries show higher median age of presentation which was reported to be 70 years old [7].Other studies reported 68 year as median age at presentation [20]. This difference can be attributed to the difference in population structure, environmental difference, genetic predisposition between Iraq and Western countries, and difference in life expectancy.

The male to female ratio in this study was 1.2:1 which was lower than that reported by other Iraq studies [16-18], as shown in the table below. This difference may be due to the difference in sample size. But it was comparable to that of Western countries and other world studies [1,7,21]. But in all studies obvious finding of male predominance was fixed. Which might be related to genetic bases as shown by results reported by Cantu ES, McGill JR et al [22]. These results provided a genetic basis for the notion that the FISH abnormalities found underlie the phenotypic M/F sex ratio and also that they may be sex chromosomes (X and/or Y) influenced [22].

The most common presenting clinical feature of CLL in this study was splenomegaly followed by anemia, then lymphadenopathy, these results were comparable with other Iraqi workers [16-18].

Similar to the current study, results obtained by a study in Thailand [19].While other Western studies showed that the incidence of lymphadenopathy is more common than splenomegaly, anemia and hepatomegaly [3]. This might be attributed to earlier diagnosis, and sample size, in addition 5 patients in the current study received treatment. Regarding staging of the CLL cases in this study, by applying Binet staging, 85% of the patients fell within Binet stage C, which is considered as a high-risk

stage. This may be attributed to lack of regular checkup and general follow up of the people's health, so most patients are not presented until signs of advanced disease begin to appear. This percentage appears higher than other Iraqi studies. The cause may be due to the smaller sample size of the current study. While Western studies showed decreasing percent of cases in stage C at diagnosis, and increasing percent of stage A cases at time of diagnosis, which is attributed to the regular checkup and the facilities available for early detection and diagnosis of the disease [20].

The mean Hb level was 10.98+2.84g/dl, with a range (5.6-16.5g/dl), which was close to the results obtained by other Iraqi workers(16,17,18), as shown in table 4.1. As most patients in the current study presented in advanced stage of the disease with infiltration of the bone marrow by leukemic cells , in addition to the fact that nearly all the patients in the current study were elderly and many of them suffered from chronic illnesses. These results were also comparable to Western studies [3]

The mean of platelets count was 162±71.28X109/l, and six patients (35.3%) presented with platelets count less than 100X109/l, and a range between 30.0-280.0X109/l. As 85% of the patients in the current study were within Binet stage C, the most advanced stage of the disease, which involves infiltration of the bone marrow by leukemic cells and suppression of platelets production. These results agreed with Iraqi and Western studies [3,16-18].

Table (10) The results of the current study and other Iraqi workers

Parameters	Current study	Huda,et al M.Sc thesis 2010 [93]	Shaimaa,et al M.Sc thesis 2010[94]	Abdulkareem ,et al PhD thesis2008[95]
Patients number	20	50	68	60
Age range	45-75	39-75	40-88	48-72
Mean age (year)	61.95±8.88 SD	59.2 ± 1.34	61.70 ± 11	61.4 ± 9.1
Male : female ratio	1.2:1	3:1	2.4:1	3:1
Mean Hb (g/dl)	10.98±2.84g/dl	10.52 ± 0.26	10.16 ± 6.6	10.0 ± 1.5
Mean platelet count ±SE ($x10^9$\L)	162.00±71.28	142.82±10.11	162.45 ± 96.96	149 ± 73.8
Mean lymphocyte count±SE($x10^9$\L)	46.72±30.22	110.97±14.40	104.56±113.64	96.9 ± 99.8
Most common presenting sign	splenomegaly	lymphadenopathy	Lymphadenopathy	splenomegaly
Percentage of high risk patients Binet stage C	85%	54%	64.7%	63.3%

Regarding the intensity of marker expression, the majority of patients show moderate intensity of expression for CD23, (this agreed with results of Gong JZ and coworkers who demonstrated that the majority of CLL cases showed either moderate or bright expression of CD23 [23], and moderate CD5 expression [24], regardless the stage of the disease , as evidenced no relation between the stage of the disease and the intensity of expression P>0.05. This was in contrast to Geisler, et al study, which demonstrated that low intensity CD23 was associated with shorter survival in CLL[25] Dadmarz and Cawley demonstrated an association of low intensity CD23 with more advanced stage of disease in CLL [26]. Study of additional cases is necessary to confirm this association. The current study is somewhat limited, however, by relatively short follow up periods, small sample size. This issue will be readdressed after long

follow-up. Regarding the relation of the marker expression to the clinical and hematological parameters, there was a significant relation between CD5 and absolute lymphocyte count (P<0.05), this passed in agreement with other Western studies , which showed that greater CD5 antigen expression ,with higher peripheral blood lymphocyte count [1].

While in the current study CD23 percent of expression showed no correlation with peripheral blood lymphocyte count, other Western studies like Jurisic et al [1], showed that no correlation was found between CD23 expression in the patients with peripheral blood lymphocytes less than $100x10^9$/L, as the patients were still in early stages of the disease with low lymphocyte count and no cell membrane changes regarding expression of molecules had been occurred yet, while the patients with peripheral blood lymphocytes >$100X10^9$/L exhibited negative correlation with very low percentages of CD23 expression, as an increase in the lymphocyte count and an accumulation of anergic B cells have been associated with marked membrane molecule alterations [27,28]. These molecules are often functionally altered and diversely expressed in comparison to those of normal cells, show different cell membrane densities, differing receptor avidity and receptor saturation, and with the expression of activation molecules [26].This is contrary to the suggestion that advanced disease is associated with higher levels of CD23 expression, based on the measurement of elevated serum levels of soluble cleaved CD23 molecules using ELISA techniques [29,30], these soluble CD23 molecules (sCD23) resulting from the spontaneous proteolysis, cleavage and release of one of the two isoforms of the trans-membrane CD23 molecule [31].

So based on the findings of the current study , and the different findings of the different studies, we conclude that CD23 is not uniformly expressed by lymphocytes in CLL patients, and its expression may depend on number of clinical parameters like stage of the disease, absolute lymphocyte count, high lymphocyte count >$100x10^9$/l .

The current study showed that were no correlation between CD38 expression and the age of the patients , WBC count, lymphocyte count and platelets count , whereas there was a relation between CD38 expression and the Hb level(P<0.05) as low level of Hb was seen with higher CD38 expression. This may be explained as low Hb levels are usually associated with advanced stages of the disease, and this pass with the positive expression of CD38, which is a poor prognostic factor. This was in agreement with the studies of other workers, like Ibrahim S, Keating M, et al[4].

The current study showed that there is a significant correlation between CD5, and CD23 expression as (P<0.05), indicating a predominance of the B cell subpopulation within the pool of the circulating lymphocytes. This was in agreement with other workers studies, like, Jurisic V, et al. Who showed that with high expression of CD23, there was also a high percentage of CD5 expression (P<0.05), which was positively correlated [(1)].

Also there was a correlation between the percent of expression of CD38+ cells and the expression of CD23 (P<0.05). This was in agreement with the results of other workers, like Poeta GD, Maurillo L, et al [(32)]. Who showed that high CD38 expression associated with higher CD23 expression emphasizes that CD38+ CLL cases are authentic B-CLL showing a greater disease activity as CD38 had been reported to play a complex role in lymphocyte proliferation [(32)].

Conclusions

1-There is a significant correlation between CD5 expression and absolute lymphocyte count, so higher peripheral blood lymphocyte associated with greater CD5 antigen expression.

2-Thereis a significant correlation between CD5, and CD23 expression, so high CD5 expression associated with high CD23 expression.

3-There is a significant negative correlation between CD38 expression and Hb level that reflects a prognostic significance.

4-There is a correlation between CD38 expression and CD23 expression.

5-No correlation between the intensity of expression of CD5, CD23, and CD38 and stage of the disease.

6-No correlation between CD38 expression and age, WBC count, and lymphocyte count.

Recommendations

1-Application of flow cytometry on larger sample size, and on other B-cell markers, including CD79b, FMC7, sIg, to show the complete scoring of CLL.

2-Depending on CD19 measuring as a gating step for B-lymphocytes in stead of the forward and side scatter characteristics, as it is more specific for B-lymphocytes.

3-Comparing the expression of CD38 before and after treatment of the patients with chemotherapy.

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