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Research Article

Immunophenotypic Profile of Patients with Clinically Diagnosed Chronic Lymphocytic Leukemia

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Abstract

Background: The diagnosis of Chronic Lymphocytic Leukemia (CLL) is based on cell morphology, clinical manifestations and immunophenotyping. Immunophenotyping by Flow cytometry is the most reliable methodology for the diagnosis of CLL which is a clonal mature B-cell neoplasm (MBCN). This study aims to evaluate the application of multiparametric Flow Cytometry Immunophenotyping (FCI) as a standard methodology for the confirmation of or exclusion of CLL diagnosis in clinically suspected CLL patients.

Methods: Four color FCI method was used in EDTA peripheral blood samples taken from 50 patients diagnosed preliminary as CLL through clinical data, complete blood count, peripheral blood film and bone marrow examination. The following fluorescent monoclonal antibodies were used: CD19, CD5, CD20, CD22, CD23, CD79b, FMC7, Kappa and Lambda light chains, CD200, CD123, CD10, CD11c, CD3, CD7, CD25, CD30, CD56, CD95, BCL2, CD34. FCI analysis was performed on a Beckman coulter Cytomics FC500 flow cytometer using software CXP to analyze data.

Result: Among 50 patients, 8(16%) showed normal T-cell population, while 42(84%) showed pathological B-cell lines. From these, 32 (64%) of 42 patients expressed typical CLL markers, whilst 10 (20%) of them showed B-cell prolymphocytic leukemia (B-PLL) profile. Scoring system proposed by Matutes., et al. and Moreau., et al. using CD5, CD23, CD22, FMC7, CD79b and SmIg (CLL, score 4-5 and other MBCN, score ≤ 3) differentiated CLL from B-PLL. CD200 was expressed in all (100%) CLL but not in B-PLL cases except 2(20%). Sensitivity and specificity of CD200 was 100% and 90% respectively.

Conclusion: FCI is a fundamental laboratory method without which final diagnosis of CLL can't be established. CD200 expression is pivotal to MBCN classification. Expression of CD200 in CLL and other MBCNs, supports inclusion of this marker in flow cytometric panels for the differential diagnosis of CLL.

Keywords: Chronic Lymphocytic Leukemia; Flowcytometry; Immunophenotyping; Monoclonal Antibodies; Mature B-cell Neoplasms; CD200

Background

Chronic lymphocytic leukemia (CLL) is distinguished from Small lymphocytic lymphoma (SLL) by its leukemic appearance which is described by the World Health Organization (WHO) classification of hematopoietic neoplasms [1]. It is the most common type of leukemia, comprising 30% of all leukemias and 80% of all chronic lymphoproliferative disorders in the United states and Europe [2]. CLL is in the heterogenous group of small chronic B- cell lymphoproliferative disorders (SBLPD), which also includes Hairy cell leukemia (HCL) Prolymphocytic leukemia (PLL) and leukemic phase of non-Hodgkin lymphoma (NHL) which encompasses Man-

tle cell lymphoma (MCL), Marginal zone lymphoma (MZL), Splenic marginal zone lymphoma with circulating villous lymphocytes (SMZL), Follicular lymphoma (FL), Lymphoplasmacytic lymphoma (LPL) as well as Small lymphocytic lymphoma (SLL) which is essentially equivalent to CLL [3].

Lymphocytosis in peripheral blood of an adult person more than 5000/cmm without other cause, must raise the suspicion of a CLL diagnosis [4]. The diagnosis is based on cell morphology, clinical manifestations and immunophenotyping [5-7]. The role of flow cytometry is to confirm the clonality of circulating B lymphocytes [6]. The characteristics of leukemia cells in the blood smear are small, mature lymphocytes with a thin layer of cytoplasm and a dense nucleus without discernible nucleoli and having partially aggregated chromatin [8]. These cells may be mixed with prolymphocytes, which may be >55% of the blood lymphocytes, that would favor a diagnosis of prolymphocytic leukemia (B- cell PLL) [9]. Immunophenotyping by Flow cytometry is the most reliable methodology for the diagnosis of CLL [10]. FCI in combination with microscopy of peripheral blood is needed to establish the diagnosis of CLL [11]. This study aims to evaluate the application of multiparametric Flow Cytometry Immunophenotyping (FCI) as a standard methodology for the confirmation of or exclusion of CLL diagnosis in clinically suspected CLL patients.

Methods

This retrospective study analyzed 50 untreated patients diagnosed preliminary as CLL through clinical data, complete blood count, peripheral blood film and bone marrow examination, referred for standard diagnosis by flowcytometric immunophenotyping (FCI) to the laboratory of Microbiology and Immunology department of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh, during the periods from January 2017 to September 2019. Data were extracted from the database and anonymized. After receiving, the peripheral blood samples were examined as soon as possible and the immunophenotyping were done without prior knowledge of clinical and hematological data. Demographic and hematological data were analyzed other than immunophenotypic characteristics. Flow cytometry results were compared with clinical data. The patients were classified into different clinical CLL stages as following the RAI classification [12,13]. The patients presented with a blood lymphocytosis more than 5000/mm^3 and /or a bone marrow lymphocytosis were defined as having low-risk disease or RAI stage 0. Patients with lymphocytosis, enlarged nodes in any site, splenomegaly and/or hepatomegaly were defined as having intermediate-risk disease (RAI stage I or stage II). Patients with disease related anemia (as defined by a hemoglobin level <11g/dl; stage III) or thrombocytopenia (as defined by a platelet count <100×10^9/l; stage IV) were defined as) high-risk disease.

Fluorescently Labeled Antibodies and Isotype control studies

The four color flow cytometric analysis was performed on a Beckman Coulter cytomics FC500 flow cytometer using software CXP to analyze data. The specific fluorescently labeled anti-human monoclonal antibodies used in this study were obtained from Abcam Biotechnology Company, Beckman Coulter and Becton Dickinson (BD**).**Monoclonal Antibodies used were: CD45-ECD, CD19-PE, CD3-PECy5, CD20-PECy7, CD30-FITC, CD95-PE, CD5-FITC, CD22-FITC, CD23-PE, CD79b-PE, Bcl-2-FITC, FMC7-FITC, CD10-PECy7, CD25-PECy7, CD7-FITC, CD56-PECy5, CD200-PE, CD123- PE, CD34-PE, Kappa-PE, Lambda-FITC. Marker of immaturity CD34 was added to exclude blast cells. Defining 4-color FC tube was used in this study. Appropriate isotype control studies to determine background fluorescence were also used.

Flow cytometry analysis and interpretation

EDTA peripheral blood samples were processed as soon as possible mostly within 2-3 hours of collection for better result. A "stain, lyse and wash" technique was used for processing of samples by optilyse C reagent (Beckman-Coulter, France).

For identification of surface markers

100ùl of sample was taken in each tube to ensure approximate concentration of 10 /ml. 2ml Beckman-Coulter lysing solution was taken in each tube, vortexed and incubated in dark at room temperature for 10-20 minutes. Then the cells were spuned at 200-300g for 3-5 minute and supernatant fluid was discarded. Cells were washed with sheath fluid, vortexed, spuned and supernatant was discarded. Pre titrated volume of flurochrome antibody were added in each tube, vortexed, incubated in dark at room temperature for 10-15 minutes, washed twice with sheath fluid, vortexed,

spuned and supernatant discarded. Cells were resuspended in 0.5 ml sheath fluid or PBS with 2% paraformaldehyde. Then the prepared samples were run on a precalibrated flow cytometer. For identification of intracellular markers pre titrated volume of surface antibody CD45 and CD19 was added into the tubes before adding lysing solution. After lysing, vortexing and incubating, permeabilizing solution was added and incubated in dark at room temperature.

The mature lymphocyte gating strategy included using dot plots of CD45 expression versus side scattering (SSC) and CD19 versus SSC and also a second gating strategy using forward scattering (FSC). A total of 30,000 events were acquired in target gate. Any antigen maker was considered positive if 20% or more of the cells reacted with a particular antibody. Data acquisition and analysis was done using software CXP.

Monoclonal B-cell population was detected in the peripheral blood according to overall sIgk:sIgλ ratio (>3 for sIgk monoclonality or <0.3 for sIgλ monoclonality) [14]. Double platform methodology was used for calculating B-cells and T-cells absolute counts [15]. Percentage of CD19+ B lymphocytes and percentage of CD3+ T lymphocytes detected by flow cytometry, were combined with the absolute leukocyte count and lymphocyte differential. According to standard criteria diagnosis was established [6,10]. Data were expressed as mean and median values and interquartile range. The groups were compared using Chi-square test, p value <0.05 was considered as Statistically significant.

Results

Patients characteristics

The demographic and hematological characteristics of CLL and B-PLL patients are shown in table 1. The age range of the patients were from 45 to 83 years (mean: 60.19 years) in CLL group and 54 to 81 years (mean: 67.80 years) in B-PLL group. Population of male dominated in both groups. This study showed significant differences between CLL and B-PLL groups in other hematological characteristics ($p < 0.05$).

Classification of CLL patients according to clinical stages by RAI is shown in figure 1. Out of 32 CLL patients diagnosed, 10 (31.25%) were in clinical stage 0 (low-risk disease), 8(25%) in stage I and 4 (12.5%) in stage II [intermediate-risk disease], 4 (12.5%) in stage III and 6 (18.75%) were in stage IV [high-risk disease].

Immunophenotypic characteristics of the study group

Of 50 patients, 8 (16%) showed normal T-cell population (CD3+, CD5+, CD7+), and 42(84%) showed abnormal B-cell lines. From these, 32 (64%) of 50 patients expressed typical CLL markers (CD19+,CD5,CD23, FMC7, Kappa or Lambda light chain restriction) whilst 10 (20%) of them showed B-cell prolymphocytic leukaemia profile (CD19+,CD5+/-,CD23-,FMC7+, Kappa or Lambda light chain restriction) (Figure 2,3,4).

Characteristics		Group of patients		P-value
		CLL (n = 32)	B-PLL (n = 10)	
Sex(n)	Male	22	8	<0.0001
	Female	10	2	
	Ratio	2.2/1	4/1	
Age(years)	Mean ± SD	60.19 ± 9.74	67.80 ± 10.83	<0.0001
	Median	61	70	
	Range	45-83	54-81	
Hemoglobin (g/dl)	Mean ± SD	10.50 ± 2.26	12.60 ± 2.41	0.015
	Median	11	12	
	Range	5-13	10-16	
WBC count ($\times 10^9$/l)	Mean ± SD	89.06 ± 144.02	66.00 ± 37.28	0.010
	Median	40	76	
	Range	5-464	13-107	
Platelets ($\times 10^9$/l)	Mean ± SD	222.63 ± 111.74	124.00 ± 23.49	0.001
	Median	236	120	
	Range	55-400	100-160	
Peripheral blood lymphocyte (%)	Mean ± SD	79.14 ± 11.22	73.40 ± 16.88	0.020
	Median	80	76	
	Range	52-97	48-93	
Bone marrow lymphocyte (%)	Mean ± SD	68.75 ± 10.75	64.80 ± 5.22	0.012
	Median	70	65	
	Range	5-93	57-70	

Table 1: Demographic and hematological characteristics of CLL and B-PLL patients (n = 42).

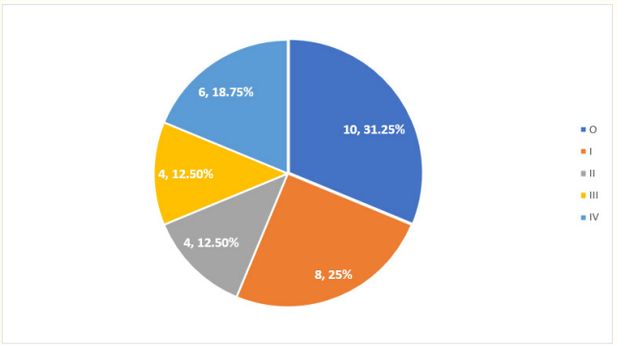


Figure 1: Distribution of CLL patients according to the clinical stages by RAI (n = 32).

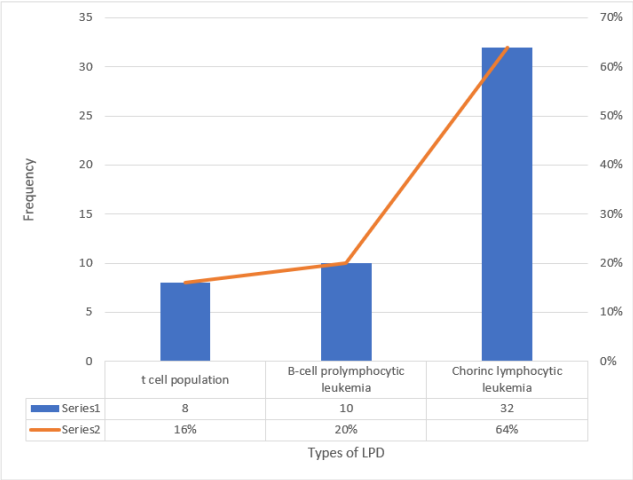
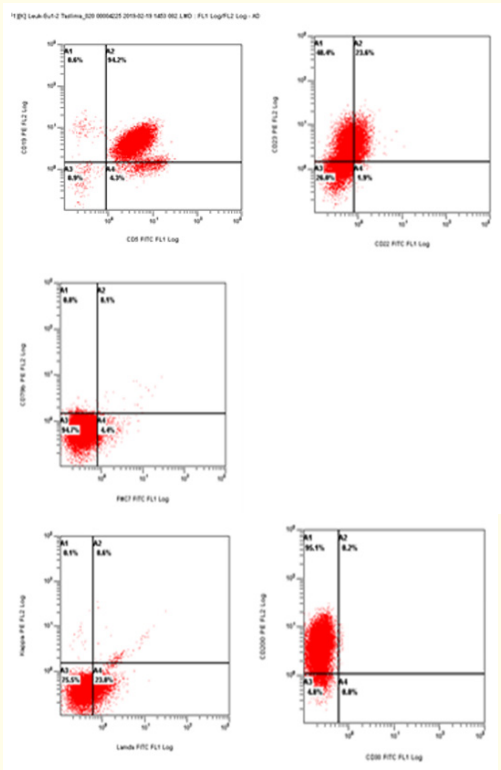


Figure 2: Flow cytometry immunophenoypic findings of clinically diagnosed chronic lymphocytic leukemia cases (n = 50).



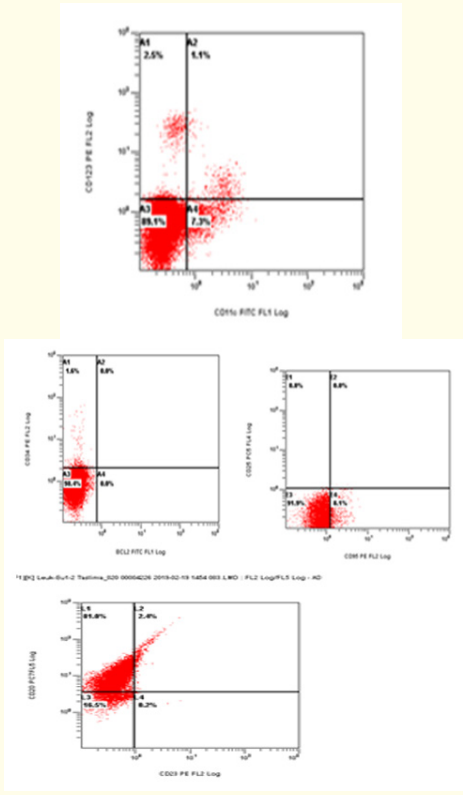
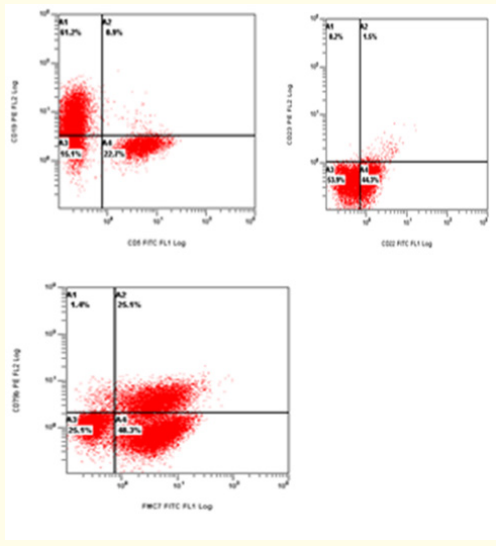


Figure 3: Flow cytometry dot plots that illustrate specific immunophenotypic profile of the chronic lymphocytic leukemia (CLL) cells.



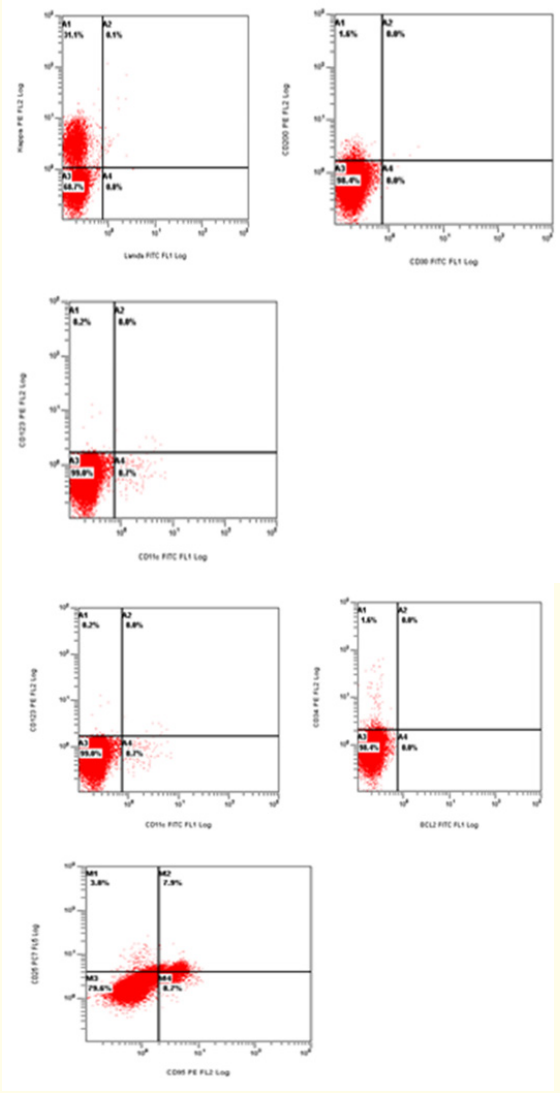


Figure 4: Flow cytometry dot plots that illustrate specific immunophenotypic profile of the B-cell prolymphocytic leukemia (B-PLL) cells.

Immunophenotyping data for each clinically diagnosed CLL patients were analyzed using CLL scoring system as proposed by Matutes., *et al.* and Moreau., *et al.* [7,10]. The total score was calculated as a score of 1 for positive reactivity toCD5, CD23, weak smIg reactivity and non reactivity of FMC7, CD79b and CD22. According to immunological diagnosis the patients were divided into two groups: immuno dg CLL (score 4-5) (32/50 pts) and immune dg B-PLL (score ≤3) (10/50 pts). Immunophenotypic characteristics of these groups are shown in Table (Table 2,3,4). CD19 was expressed in all CLL and B-PLL patients. Among 32 cases of CLL, CD5 was positive in 93.75% (30 of 32 cases), CD23 positivity was seen in 81.25% (26 of 32 cases), FMC7 negativity in 87.50% (28 of 32 cases), smIg weak positivity in 93.75% (30 of 32 cases), CD20 positive in 25% (8 of 32 cases). All CLL cases were CD200 positive 100% (32 of 32 cases). All 32 cases were negative for CD11c and CD123.

Markers	Points	
	1	0
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
sIg	Low	Medium/High
CD22/CD79b	Low/Negative	Medium/High

Table 2: Scoring system for the differential diagnosis of Chronic Lymphocytic Leukaemia (Matutes., *et al.* 1994/2010).

Antigen	%n/N		P-value
	CLL (score4-5)	B-PLL (scores≤ 3)	
CD19 positive	100(32/32)	100(10/10)	NS
CD5 Positive	93.75(30/32)	60 (6/10)	<0.0001
CD23 Positive	81.25(26/32)	0 (0/10)	NS
smIg weak positive	93.75(30/32)	20(2/10)	<0.0001
FMC7 negative	87.50(28/32)	40(4/10)	<0.0001
CD22 negative / weak positive	93.75(30/32)	40(4/10)	<0.0001
CD79b negative	100(32/32)	20(2/10)	NS
CD20 positive	25(8/32)	100(10/10)	NS
CD200 positive	100(32/32)	20(2/10)	NS
CD11c negative	100 (32/32)	100(10/10)	NS
CD123 negative	100(32/32)	100(10/10)	NS

Table 3: The frequency of antigen expression in patients with CLL (score 4-5) and B-PLL (score ≤ 3).

n = Number of patients that exhibit specific pattern of the antigen expression; N = Total number of the analyzed patients; p-value= CLL VS. B-PLL; NS = Not Significant

Pattern of Antigen Expression		%n/N		P-value
		CLL(score 4-5)	B-PLL(Score <3)	
CD5	Dim	6.25(2/32)	40(4/10)	0.353
	Moderate	56.25(18/32)	40(4/10)	
	Bright	37.50(12/32)	20(2/10)	
CD23	Dim	18.75(6/32)	100(10/10)	NS
	Moderate	68.75(22/32)	0(0/10)	
	Bright	12.50(4/32)	0(0/10)	
CD22	Dim	93.75(30/32)	40(4/10)	<0.0001
	Moderate	6.25(2/32)	20(2/10)	
	Bright	0(0/32)	40(4/10)	
CD79b	Dim	100(32/32)	20(2/10)	NS
	Moderate	0(0/32)	20(2/10)	
	Bright	0(0/32)	60(6/10)	
FMC7	Dim	87.50(28/32)	40(4/10)	<0.0001
	Moderate	12.50(4/32)	20(2/10)	
	Bright	0(0/32)	40(4/10)	
sIg	Dim	93.75(30/32)	20(2/10)	<0.0001
	Moderate	6.25(2/32)	20(2/10)	
	Bright	0(0/32)	60(6/10)	

Table 4: The frequency of antigen expression patterns in group with CLL (score 4-5) and B-PLL (score<3).

n = Number of patients that exhibit specific pattern of the antigen expression; N = Total number of the analyzed patients; NS = Not Significant, p value = CLL vs. B-PL.

Out of 10 cases of B-PLL, CD5 positivity was seen in 60% (6 of 10 cases), all 10 cases were CD23 negative, FMC7 was positive in 60% (6 of 10 cases), medium to bright smIg positivity in 80% (8 of 10 cases), CD79b positivity in 80% (8 0f 10 cases), CD22 positivity in 60% (6 of 10 cases), CD20 positivity in 100% (10 of 10 cases). CD200 were negative in 80% (8 of 10 cases), but all cases were negative for CD11c and CD123.

It has been revealed that both CD5 antigen and CD23 antigen were expressed with higher frequency in CLL group than B-PLL group (93.75% vs. 60%, p <0.0001 and 81.25% vs. 0%, p >0.05, respectively). CD5 antigen expression patterns showing that pattern $CD5^{+moderate/bright}$ predominated in CLL group (p = 0.353). In case of CLL, CD23 showed higher expression patterns ($CD23^{+moderate}$ and $CD23^{+bright}$) comparing to B-PLL cases which were characterized mainly with $CD23^{+dim}$ expression pattern (p > 0.05).There was $FMC7^{+dim}$expression pattern in CLL cases (87.5%) comparing to B-PLL which showed $FMC7^{+moderate/bright}$ (60%) (p < 0.0001). In CLL cases frequency of expression of CD20 was in 25% of cases comparing to B-PLL cases which showed CD20 in 100% of cases (p > 0.05). $CD22^{+dim}$ expression pattern was higher in CLL cases comparing to B-PLL cases (93.75% vs. 40%, p < 0.001). CD79b+dim expression pattern was in all CLL cases comparing to B-PLL cases (100% vs.20%, p > 0.05).

Presence of light chain restriction defines the monoclonality of B-cell population, which was detected in all CLL and B-PLL cases. The majority of CLL cases had sIg^{+dim} expression.

CD200 was expressed in all (100%) CLL cases compared with B-PLL cases which expressed in two (20%) cases. The median percent expression was 90.60% (range 47.2 -99.2%), with an MFI 8.87 (range 2.18- 20.6) in CLL compared with 6.85% (range 1.1- 37.2%) with an median fluorescence intensity (MFI) 1.78 (range 1.24-3.45) in B-PLL cases (p < 0.001) (Table 5).

	CLL group		B-PLL group		
	Mean ± SD	Median (Range)	Mean ± SD	Median (Range)	P-value
CD200(%)	88.10 ± 10.30	90.60 (47.2-99.2	11.25 ± 12.00	6.85 (1.1-37.2)	0.001
CD200(MFI)	8.96 ± 3.92	8.87 (2.18-20.6	1.90 ± 0.66	1.78 (1.24-3.45)	0.010

Table 5: CD200 marker positivity in chronic lymphocytic leukemia (CLL) and B-cell prolymphocytic leukemia (B-PLL).

In both CLL and B-PLL groups the cut-off value of CD200 expression on malignant clone cells was 30.40%, where it showed 100% sensitivity, 90%specificity, 96.75% positive predictive value, and 100% negative predictive value (Table 6).

Criterion	Sensitivity	Specificity	PPV	NPV
>30.40	100.00	90.00	96.75	100.00

Table 6: Sensitivity and specificity of CD200 (%).

PPV: Positive Predictive Value; NPV: Negative Predictive Value.

Discussion

For the diagnosis and subclassification of Mature B-cell neoplasm (MBCN), particularly CLL, immunophenotyping by flow cytometry is essential [16,17]. A precise diagnosis is very important as the clinical evolution and response to treatment differ between CLL and other MBCN. Classical cytomorphology, FCI, immunohistochemistry or cytogenetic methods are needed to establish an accurate diagnosis [18,19]. It becomes very difficult to implement these methods because of high cost and lack of skilled personel, limited financial and technological resources. As the most reliable methodology for the diagnosis of CLL is immunophenotyping by flow cytometry, we studied clinically diagnosed 50 consecutive patients referred to our laboratory to see the impact of implementation of FCI for precise diagnosis.

Comparing to other MBCN, CLL has a relatively specific immunophenotypic profile but there is some overlapping regarding the expression of membrane markers. The CLL scoring system was created in order to make distinction between CLL and other MBCN entities easier [20,21]. The revised Matutes score system, based on the immunophenotypic analysis of five membrane markers (CD5+, CD23+, FMC7-, and negative/weak expression of surface immunoglobulin and CD79b), was employed to diagnose CLL cases [21]. Out of 50 analyzed cases, 32 (64%) cases were diagnosed as CLL which were defined by a score ≥4; 10 (20%) B –PLL cases were identified by a score ≤ 3. At presentation, the CLL patients had anemia and /or thrombocytopenia with typical clinical and hematological characteristics. CLL patients had significantly higher mean values of platelets in comparison to B-PLL patients, similar results has been found in another study [7]. Both CLL and B-PLL cases had absolute count of monoclonal B cells above 5000 cells/ùl. In B-PLL cases there was > 55% prolymphocytes in peripheral blood compared to CLL cases where prolymphocytes were up to 10%. Diagnosis of B-PLL was considered in cases with high lymphocyte counts associated to >55% prolymphocytes in peripheral blood, splenomegaly, absence of lymphadenopathy and no previous history of MBCN.

The expression of three B-cell lineage antigens (CD19, CD20, CD22) and five antigens from the CLL scoring system (CD5, CD23, FMC7, CD79b, sIg), was analyzed to evaluate the value of CD markers in the differential diagnosis of CLL. The B cell lineage marker CD19 was expressed in all CLL and B-PLL cases which is the only marker consistently expressed on Leukemic B-cells. This finding is consistent with another study which used CD19 for immunophenotypic analysis of MBCN, including CLL as one of the best gating antigen [22]. We found that CD20 and CD22 expression were consistently lower on CLL cells because of their low expression levels on CLL cells. In contrast, in case of B-PLL, all cases showed bright expression of CD20 and 60% of cases CD22. Expression of CD20 was not important for differential diagnosis of CLL, because CD20 was not expressed in all CLL cases in this study. Similar result has been reported by another study [23]. The assessment of CD20 expression is important where anti- CD20 immunotherapy is planned.

CLL cells have immunophenotypic signature similar to that of activated follicular marginal zone B1 cell, which normally express CD5, and coexpress membrane IgM and IgD [24]. CD5 is a prerequisite marker for the diagnosis of CLL [25,26]. In pathological condition, CD5 is usually expressed in CLL, one third of B-PLL, MCL, in 10% of diffuse large B-cell lymphoma (DLBCL) [16]. In this study 93.75% CLL cases were CD5 positive whereas B-PLL cases were positive in 60% cases. The frequency and intensity of CD5 expression was higher in CLL compared to B-PLL. B-PLL can be divided into CD5+ B-PLL arising in CLL/SLL) and CD5- B-PLL (de novo PLL). CD5+ B-PLL has a longer median survival than CD5- B-PLL. Several other studies found the frequency of CD5 expression was very high in CLL [27,28]. Expression of CD5 is almost universal in CLL/SLL and MCL, therefore detection of CD5 is useful in the diagnosis of these neoplasms [29].

The monoclonality of B-cells is determined by the presence of sIg light chain restriction using flow cytometry. In CLL cases monoclonality was detected in all cases in this study, and distribution of $sIgk^+$ and $sIg\lambda^+$ were equal.

Matutes., *et al*. showed in their study that sIg was expressed in 92% of CLL cases with similar distribution of $sIgk^+$ and $sig\lambda^+$ [20]. In contrast, B-PLL cases showed medium to bright expression of sIg light chain except in one case which expressed weak sIg. Out of 10 B-PLL cases in this study, 6(60%) showed $sIgk^+$ light chain restriction and 4(40%) showed $sIg\lambda^+$ light chain restriction. The findings of this study revealed that almost all CLL cases had sIg^{+dim} expression pattern compared to B-PLL cases, which is important for differential diagnosis of CLL.

FMC7 is a glycoprotein found on circulating B lymphocytes. FMC7 identifies the same cell type as CD22, but is a superior alternative for characterization of B-cell lymphomas. It is negative to weakly positive in CLL but strongly positive in B-PLL, HCL, MCL and SLVL. FMC7 is thereby used for the differentiation of B-PLL from CLL (e.g., progression of CLL to B-PLL) and for the confirmation of the diagnosis of other MBCN, such as HCL, MCL and SLVL [30]. CD23 is a low affinity IgE receptor, expressed on the surface of IgM and IgD expressing B-cells and also found on monocytes, minor subset of T cells, platelets eosinophils, Langerhans cells and follicular dendritic cells [31]. Differential diagnosis among CLL/SLL, MCL and MZL is best demonstrated by expression of CD23 and FMC7. CLL/SLL usually is CD23+ and FMC7-, but MCL and MZL are consistently CD23- and FMC7+ [32]. This pattern of expression was useful for detecting B-cell prolymphocytic leukemia (B-PLL) and prolymphocytic transformation in CLL (CLL/PL) as well in a study by Ahmed., *et al*. [33]; most of these cases were CD23- and FMC7+. Similar findings were observed on B-PLL and CLL/PL cases in other studies [34,35].

A subgroup of CLLs with trisomy 12, may have CD23± and FMC7+ expression pattern, which are at greater risk for a more aggressive course [36]. In another study expression of low CD23 and high FMC7 was associated with short survival [37]. In this study all CLL cases were CD23+ and FMC7 -. It is the distinguishing marker for CLL from MCL which consistently shows expression pattern of CD23-FMC7 +. In contrast, all of B-PLL cases were CD23- FMC7+. So it is challenging to differentiate B-PLL from leukemic variant of MCL which consistently shows this expression pattern. As MCL can have expression pattern of CD23 and FMC7 like CLL or B-PLL, so it is important to diagnose MCL precisely by flow cytometry and to be confirmed by detecting cyclin D1 by immunohistochemistry and/or by detection of chromosomal translocation t (11;14).

CD79b represents the latest antigen that was incorporated into CLL scoring system [19]. CD79b is one of the B-cell receptor complex component. In the majority of the typical CLL, it has been observed that CD79b is greatly diminished or even absent [38,39]. In this study, $CD79b^{+low}$ expression pattern was detected in all CLL cases compared to B-PLL cases (100% vs. 20%). This finding is consistent with a study which detected $CD79b^{+low}$expression pattern in majority of CLL cases [39]. These characteristics of CD79b make it a good marker for differential diagnosis of CLL.

CD 200 (previously referred to as OX2) is a membrane glycoprotein belongs to immunoglobulin superfamily and has an immunosuppressive role; expressed on a subset of T-cells, all CD19+ B- cells but not Natural killer cells, monocytes, granulocytes or platelets. It is also expressed on human plasma cells, myeloma and CLL cells [40]. When CD23 is not expressed by the leukemic cells or in cases of MCL expressing CD23, the diagnosis of CLL becomes challenging. Flow cytometry enables the differentiation of CLL from MCL in majority of cases [41]. This study investigated the expression of CD200 in clinically diagnosed CLL patients. It was expressed in all (100%) CLL cases with moderate intensity compared with B-PLL, where only 2(20%) cases expressed CD200 ($p > 0.05$). It has been suggested that it is a good marker to differentiate from CLL but not from MCL cases with increased number of prolymphocytes. In cases with Matutes score ≤ 3, CD200 expression is pivotal to MBCN classification. Whenever CD200 is positive, the differential diagnosis among atypical CLL and CD5+ NHL should be considered. In this situation the Matutes score associated with the quantification of prolymphocytes by morphology will guide the classification. A study reported almost similar findings which showed that all typical and atypical CLL cases were CD200 positive but almost all B-PLL cases did not express CD200 except one B-PLL case which presented with high expression of CD200 ; all MCL cases were negative for CD200 [42]. In this study CD200 is expressed uniformly in CLL

which confirms the similar studies [43,44]. To distinguish among CLL, B-PLL and MCL inclusion of CD200 in flow cytometry routine panels could be useful.

In this study, CD11c and CD123 were used to differentiate CLL from HCL which are considered to be marker of HCL. All CLL and B-PLL cases did not express these markers, moreover CD25 was negative in these cases which further exclude HCL.

The most frequent associations are CD5+/CD10-, whose main hypotheses are CLL and MCL. In this study all CLL and B-PLL cases are CD10 –, which could arise the question of MCL positivity but Bcl2 negativity of CLL and B-PLL further exclude MCL as it is usually Bcl2 positive. A study formally demonstrates that CLL and B-PLL are two distinct diseases, each showing a specific gene expression. B-PLL has a homogenous genomic profile irrespective of its heterogenecity in laboratory features [45]. B-PLL has shown poor response to CLL therapy.

Conclusion

Results of this study have shown that specific immunophenotype which differentiates CLL from B-PLL with the following marker combination : CLL – CD19+CD5$^{+bright}$ CD23+ FMC7 – sIg^{+dim} CD22$^{+dim/-}$ CD79b$^{+dim/-}$ CD20^{+dim} ; while B-PLL--CD19+ CD5+/- CD23- FMC7+ sIg$^{+bright}$ CD22$^{+bright}$ CD79b$^{+bright}$ CD20$^{+bright}$. It has also been found that these antigens can not separately differentiate CLL from B-PLL, combination of these antigens makes it possible to distinguish them. Scoring system using CD5, CD23, CD22, FMC7, CD79b and SmIg was found to be useful in differential diagnosis of CLL. Expression of CD200 in CLL and other MBCNs, giving further support for the inclusion of this marker in flow cytometric panels for the differential diagnosis of CLL. FCI in combination with cytomorphology can distinguish B-PLL from CLL which are two distinct diseases and thereby can help in treatment plan.

Conflicts of Interest

The author has no conflicts of interest to declare.

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Bibliography

1. Jaffe ES., *et al*. "World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues". Lyon, France: IARC Press (2001): 127-130.

2. Foucar K. "Bone marrow pathology". In: Chronic lymphoproliferative disorders. Chicago: ASCP Press (1995).

3. Huang JC., *et al*. "CD5-negative B-cell leukemias are rarely classifiable as chronic lymphocytic leukemia". *American Journal of Clinical Pathology* 111 (1999): 123-130.

4. Chiorazzi N., *et al*. "Chronic Lymphocytic Leukemia". *The New England Journal of Medicine* 352 (2005): 804-815.

5. Swerdlow SH., *et al*. "World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues". Lyon: IARC Press (2008): 180-182.

6. Hallek M., *et al*. "Guidelines for the diagnosis and treatment of chronic Lymphocytic leukemia: a report from the international workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute- Working group 1996 guidelines". *Blood* 111 (2008): 5446-5456.

7. Lin TS., *et al*. "Chronic Lymphocytic Leukemia". In: Hoffman R, Benz. Jr EJ, Shattil SJ, Furie B, Silberstein LE, McGlave P, *et al*, editors. Hematology: Basic Principles and Practice. 5th ed. Churchill livingstone/Elsevier Philadelphia, USA (2008): Ch.83.

8. Gribben JG. "Molecular profiling in CLL". *Hematology: the American Society of Hematology Education Program* (2008): 444-449.

9. Melo JV., *et al*. "The relationship between chronic lymphocytic leukemia and prolymphocytic leukemia: IV. Analysis of survival and prognostic features". *British Journal of Haematology* 63 (1986): 377-387.

10. Bennet JM., *et al*. "Proposal for the classification of chronic (mature) B and T lymphoid leukemias". *Journal of Clinical Pathology* 42 (1989): 567-584.

11. Bain BJ. "Blood cells. A Practical Guide". Blackwell Publishing Limited. (2006): 439.

12. Rai KR., *et al*. "Clinical staging of chronic lymphocytic leukemia". *Blood* 46 (1975): 219-234.

13. Rai KR. "A critical analysis of staging in CLL". In: Gale RP, Rai KR, editors. Chronic Lymphocytic Leukemia: Recent Progress and Future Directions. New York, NY: Liss (1987): 253-264.

14. Marti G., *et al*. "Diagnostic criteria for monoclonal B-cell lymphocytosis". *British Journal of Haematology* 130 (2005): 325-332.

15. D'Archangelo M. "Flow cytometry: new guidelines to support its clinical application Enumeration of Immunologically Defined Cell Populations by Flow Cytometry Approved Guideline, 2nd ed". Cytometry 72B (2007): 209-10.

16. Muller Hermelink HK., *et al*. "Chronic lymphocytic leukemia/small lymphocytic lymphoma". In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC Press (2008): 180-182.

17. Matutes E., *et al*. "Diagnostic issues in chronic lymphocytic leukemia (CLL)". *Best Practice and Research Clinical Haematology* 23 (2010): 3-20.

18. Habib Nk and Finn WG. "Unsupervised immunophenotypic profiling of chronic lymphocytic leukemia". *Cytometry Part B: Clinical Cytometry* 70 (2006): 124-135.

19. Orfao A., *et al*. "Diagnosis of hematological malignancies: new applications for flow cytometry". *Hematology (EHA)* 2 (2006): 6-13.

20. Matutes E., *et al*. "The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL". *Leukemia* 8 (1994): 1640-1645.

21. Moreau EJ., *et al*. "Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b)". *American Journal of Clinical Pathology* 108 (1997): 378-382.

22. Bene MC., *et al*. "Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European Leukemia Net Work Package10". *Leukemia* 25 (2011): 567-574.

23. Ginaldi L., *et al*. "Levels of expression of CD19 and CD20 in chronic B cell leukaemias". *Journal of Clinical Pathology* 51 (1998): 364-369.

24. Chorazzi N., *et al*. "Cellular origin (s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities". *Blood* 117 (2011): 1781-1791.

25. Davis BH., *et al*. "Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia: medical indications". *Cytometry Part B: Clinical Cytometry* 72B (2007): S5-S13.

26. Landgren O., *et al*. "B cell clones as early markers of chronic lymphocytic leukemia". *The New England Journal of Medicine* 360 (2009): 659-667.

27. Pangalis GA., *et al*. "B- chronic lymphocytic leukemia, small lymphocytic lymphoma and lymphoplasmacytic lymphoma, including Waldenstorm's macroglobulinemia: a clinical, morphologic, and biologic spectrum of similar disorders". *Seminar on Hematology* 36 (1999): 104-114.

28. Deneys V., *et al*. "Atypical lymphocytic leukemia and mantle cell lymphoma immunologically very close: flow cytometric distinction by use of CD20 and CD54 expression". *Leukemia* 15 (2001): 1458-1465.

29. Ballesteros E., *et al*. "CD5+ low-grade marginal zone B-cell lymphomas with localized presentation". *American Journal of Surgical Pathology* 22 (1998): 201-217.

30. Garcia DP., *et al*. "Diagnostic Usefulness of CD23 and FMC-7 antigen Expression Patterns in B-cell lymphoma Classification". *American Journal of Clinical Pathology* 115 (2001): 258-265.

31. Fournier S., *et al*. "CD23 antigen regulation and signaling in chronic lymphocytic leukemia". *Journal of Clinical Investigation* 89 (1992): 1312-1321.

32. kilo MN and Dorfman DM. "The utility of flow cytometric immunophenotypic analysis in the distinction of small lymphocytic lymphoma/ chronic lymphocytic leukemia from mantle cell lymphoma". *American Journal of Clinical Pathology* 105 (1996): 451-457.

33. Ahmad E., *et al*. "Clinical utility of CD23 and FMC-7 antigen coexistent expression in B-cell lymphoproliferative disorder subclassification". *Cytometry* 50.1 (2002): 1-7.

34. Catovsky D., *et al*. "Heterogenicity of B-cell leukemias demonstrated by the monoclonal antibody FMC-7". *Blood* 58 (1981): 406-408.

35. Stark AN., *et al*. "Prolymphocytoid transformation of CLL: a clinical and immunological study of 22 cases". *Leukemia Research* 10 (1986): 1225-1232.

36. Jennings CD and Foon KA. "Recent advances in flow cytometry: application to the diagnosis of hematologic malignancy". *Blood* 90 (1997): 2863-2892.

37. Geisler CH., *et al*. "Prognostic importance of flow cytometric immunophenotyping of 540 consecutive patients with B-cell chronic lymphocytic leukemia". *Blood* 78 (1991): 1795-1802.

38. Schlette E., *et al*. "CD79b Expression in chronic lymphocytic leukemia. Association with trisomy 12 and atypical immunophenotype". *Archives of Pathology and Laboratory Medicine* 127 (2003): 561-566.

39. Garcia vela JA., *et al*. "CD79b expression in B cell chronic lymphocytic leukemia: its implication for minimal residual disease detection". *Leukemia* 13 (1999): 1501-1505.

40. Palumbo GA., *et al*. "CD200 as a tool for differential diagnosis between B-cell chronic lymphocytic leukemia and mantle cell lymphoma". *Blood* 93 (2008): 33.

41. El-Sewefy DA., *et al*. "Flow cytometric evaluation of CD200 as a tool for differentiation between chronic lymphocytic leukemia and mantle cell lymphoma". *Egyptian Journal of Haematology* 39.2 (2014): 42-46.

42. Sandes AF., *et al*. "CD200 Has an Important Role in the Differential Diagnosis of Mature B-Cell Neoplasms by Multiparameter Flow Cytometry". *Cytometry Part B* 86B (2014): 98-105.

43. Alapat D., *et al*. "Diagnostic usefulness and prognostic impact of CD200 expression in lymphoid malignancies and plasma cell myelomas". *American Journal of Clinical Pathology* 137 (2012): 93-100.

44. Palumbo GA., *et al*. "CD200 expression may help in differential diagnosis between mantle cell lymphoma and B- cell chronic lymphocytic leukemia". *Leukemia Research* 33 (2009): 1212-1216.

45. Giudice ID., *et al*. "B-Cell Prolymphocytic Leukemia (B-PLL) and chronic lymphocytic leukemia (CLL) Express Distinct Genomic Profiles". *Blood* 106 (2005): 4372.