Original Article

Comparing flow cytometry immunophenotypic and immunohistochemical analyses in diagnosis and prognosis of chronic lymphoproliferative disorders: Experience from a Tertiary Care Center

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ABSTRACT

Background: The latest World Health Organization classification incorporates extensive description of immunophenotype of the neoplastic cells while describing chronic lymphoproliferative disorders (CLPDs). The present study was undertaken with an aim to identify and compare the roles of flow cytometry (FCM) and immunohistochemistry (IHC) as modalities of immunophenotyping in the diagnosis of CLPDs. **Materials and Methods:** Thirty untreated cases of CLPDs were enrolled in the study. Twenty eight cases of B-CLPD were divided into two groups - chronic lymphocytic leukemia (CLL) (21 patients) and non-CLL (7 patients). Peripheral blood/bone marrow aspirate samples were analysed by FCM using various panels of monoclonal antibodies. Immunohistochemical analysis of bone marrow biopsies obtained from these patients was also performed. **Results:** Panel A of monoclonal antibodies comprising CD5, CD23, CD22, surface membrane immunoglobulin (SmIg), FMC7 and Panel B comprising CD5, CD23, CD22, SmIg, FMC7, CD79b were useful ($P < 0.01$ and <0.001 respectively) while Panel C comprising CD5, CD23, SmIg, FMC7 and CD79b was not found to be useful in distinguishing CLL from non-CLL ($P > 0.05$) The concordance rate between FCM and IHC ranged from 80% to 100% for all comparable immunological markers. In all cases of CLPDs, we propose a screening panel comprising 9 markers including CD19, CD5, CD23, FMC7, CD10, CD20, CD3, kappa and lambda, which are important for specifying the lineage (B or T), to differentiate CLL from non-CLL group and for deciding the secondary panel. **Conclusion:** Scoring system using CD5, CD23, CD22, FMC7, CD79b, and SmIg is useful in differentiating CLL from non-CLL cases. Concordance rate of FCM and IHC in CLPDs is 93.3%. Using a panel comprising CD19, CD5, CD23, FMC7, CD10, CD20, CD3, kappa and lambda, a diagnosis of CLL, mantle cell, and follicular lymphoma, the three most common CLPDs can be made. Secondary panels for diagnosis of hairy cell leukemia and T-cell CLPD should be utilized.

Key words: Chronic lymphocytic leukemia, chronic lymphoproliferative disorders, flow cytometry, immunohistochemistry

INTRODUCTION

Chronic lymphoproliferative disorders (CLPDs) are a heterogeneous group of leukemias/lymphomas, which are defined by the proliferation of mature B and rarely T/NK lymphoid cells in the peripheral blood, bone marrow and/or lymph nodes/spleen and other lymphoid tissue. The present World Health Organization (WHO) classification incorporates extensive descriptions of the immunophenotype of the neoplastic cells while describing CLPDs.[1] Immunophenotyping can be done

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on fresh peripheral blood/bone marrow aspirate/lymph node aspirate samples using flow cytometry (FCM) or on formalin-fixed-paraffin-embedded tissue using immunohistochemistry (IHC). Each of the methods available comes with its advantages and disadvantages and contribution towards making the final hematological diagnosis. FCM provides a rapid, selective identification of normal and abnormal cell population excluding the dead cells and allows the detection of weakly expressed antigens. IHC on bone marrow biopsy sections, on the other hand, offers a visual impression of architectural relationships between different population of cells and to the stromal component.[2]

The present study was undertaken with an aim to identify and compare the roles of FCM and IHC in the diagnosis of CLPD.

MATERIALS AND METHODS

Thirty eight consecutive fresh untreated cases of CLPDs presenting in a tertiary care hospital were enrolled in this study conducted over a 2 years period from 2011 to 2013. Previously treated cases of CLPDs, cases with total nucleated cell count in peripheral blood/bone marrow that was insufficient for FCM or cases with inadequate length of bone marrow biopsies (<1.5 cm) and cases that did not have infiltration on bone marrow biopsy were excluded from the study. After excluding 8 such cases, 30 CLPD cases were finally included in the study.

The patient age ranged from 32 to 81 years (mean 62.1 years) and a male-female ratio of 2.7:1 was obtained. Of the 30 cases included in the study, 28 cases were B-CLPD and 2 cases were T-CLPD. The 28 B-CLPD cases were divided into two groups - chronic lymphocytic leukemia (CLL) (21 patients) and non-CLL (7 patients). The non-CLL group included three cases of hairy cell leukemia (HCL), two cases of follicular lymphoma (FL) and one case of mantle cell lymphoma (MCL) and splenic marginal zone lymphoma (SMZL). In the T-CLPD, one case of T-large granular lymphoma (T-LGL) and T-prolymphocytic lymphoma (T-PLL) were included.

Two milliliters of peripheral blood and bone marrow aspirate was collected separately in ethylene diamine tetraacetic acid tubes and was processed for FCM within 24 h. Fresh peripheral blood and bone marrow aspirate smears were prepared at the same time of collection and stained with Leishman stain for evaluation.

The FC-500 flow cytometer with CXP Software (Beckman Coulter) was used for FCM. Before acquisition os samples, calibration and fluorochrome compensation of FCM were performed according to manufacturer's instructions. Based on the cell count of the sample, it was diluted with isotone to achieve a total nucleated cell count between 5 and 10 lakh/μl. Monoclonal antibodies CD19, CD5, CD23, CD22, FMC7, kappa, lambda, CD11c, CD38, CD10, CD79b, CD25, CD3, CD4, and CD8 were used. Expression of a particular antigen by at least 20% of cells was required for considering it positive for a specific marker. Intensity of staining was measured as weak, moderate or bright depending on mean fluorescence intensity scale.

The nature of reactivity of CLL and non-CLL patients to Panel A of monoclonal antibodies which was comprising CD5, CD23, CD22, surface membrane immunoglobulin (SmIg), and FMC7, Panel B which was comprising CD5, CD23, CD22, SmIg, FMC7, and CD79b, and Panel C which was including CD5, CD23, SmIg, FMC7, and CD79b was observed.

Freshly obtained bone marrow biopsy specimens were left for fixation overnight (20–24 h) and then washed in distilled water for 30 min. Specimens were then decalcified in Gooding and Stewart's decalcification fluid (10% formic acid and 5% formaldehyde) for about 6 h before being processed and embedded in paraffin wax. Sections were stained routinely with hematoxylin and eosin, Perl's stain (for iron) and silver stain (for reticulin fibres). The infiltration pattern of biopsy was noted in each case.

For IHC, antigen retrieval was done using heat induced epitope retrieval in a pressure cooker and Streptavidin-biotin method with horse-radish peroxidase enzyme was used as detection kit (Dako, Denmark). The panel of antibodies used in bone marrow biopsy sections included CD19, CD5, CD23, CD22, CD10, kappa, lambda, CD11c, CD79b, CD25, CD3, CD4, CD8, and cyclin D1.

A Microsoft excel sheet was generated and SPSS Software version 11.5 (SPSS, Chicago, IL, USA) used to analyze the data collected. $P < 0.05$ was regarded as statistically significant.

RESULTS

Flow cytometric analysis

The mean total leukocyte count in peripheral blood was 72,800 cells/μl (range: 5100 cells/μl to 292,000 cells/μl). Percentage of gated lymphocytes in the bone marrow aspirate samples ranged from 10% to 90% (mean 71%) in all patients, 40–98% (mean 75%) in CLL patients, and 10–87% (mean 62%) in non-CLL patients.

Of the 21 cases of CLL, CD5 positivity was seen in 90.5% (19 of 21 cases), CD23 positivity was seen in 95.2% (20 of

21 cases), CD22 positivity was seen in 38% (8 of 21 cases), weak SmIg positivity in 100% cases, FMC7 positivity in 33.3% (7 of 21 cases), and CD79b positivity seen in 14.3% (3 of 21 cases) [Figure 1]. Of the 7 cases of non-CLL, CD5 positivity was seen in 28.5% (2 of 7 cases), CD23 positivity in 57.1% (4 of 7 cases), CD22 positivity in 42.8% (3 of 7 cases), weak SmIg positivity in 71.4% (5 of 7 cases), FMC7 positivity in 85.7% (6 of 7 cases), and CD79b positivity was seen in 71.4% (5 of 7 cases) [Table 1].

After assigning a score of 1 for positive reactivity to CD5, CD23, weak SmIg reactivity and nonreactivity of FMC7, CD79b, and CD22, total score was calculated as proposed by Matutes *et al*. and Moreau *et al*.[3,4] Using Panel A, a score of ≥4 was obtained in 18 of 21 CLL cases (85.7%) and a score of <4 was obtained in 85.7% (6 of 7) non-CLL cases and the results were statistically significant ($P < 0.01$). Using Panel B, a score of ≥4 was obtained in 95.2% (20 of 21) CLL cases and a score of <4 was obtained in 85.7% (6 of 7) non-CLL cases and the results were statistically significant ($P < 0.001$). Using Panel C, a score of ≥3 was obtained in 85.7% (18 of 21) CLL cases but a score of <3 in non-CLL cases was obtained in only 57.1% (4 of 7 cases) and the results were statistically insignificant ($P > 0.05$) [Table 2]. The results indicate that both Panels A and B are useful in distinguishing CLL from non-CLL, but a lower *P* value for Panel B suggests its superiority over Panel A.

Histopathological and immunohistochemical analysis

In CLL, varied patterns of bone marrow infiltrations were observed including a diffuse pattern of infiltration 4/21 cases, nodular pattern in 4/21 cases, interstitial in 5/21 and mixed pattern in 8/21 cases. In HCL, 2/3 cases had diffuse and 1/3 cases had interstitial pattern of infiltration; both cases of FL had nodular pattern; single case of SMZL had interstitial pattern; MCL case had nodular pattern of infiltration. Among the T-CLPDs, single case of T-PLL and T-LGL showed diffuse and interstitial pattern, respectively.

One of the cases of bone marrow biopsy showed neoplastic cells with typical fried egg appearance on hematoxylin and eosin, chicken wire pattern of vasculature on

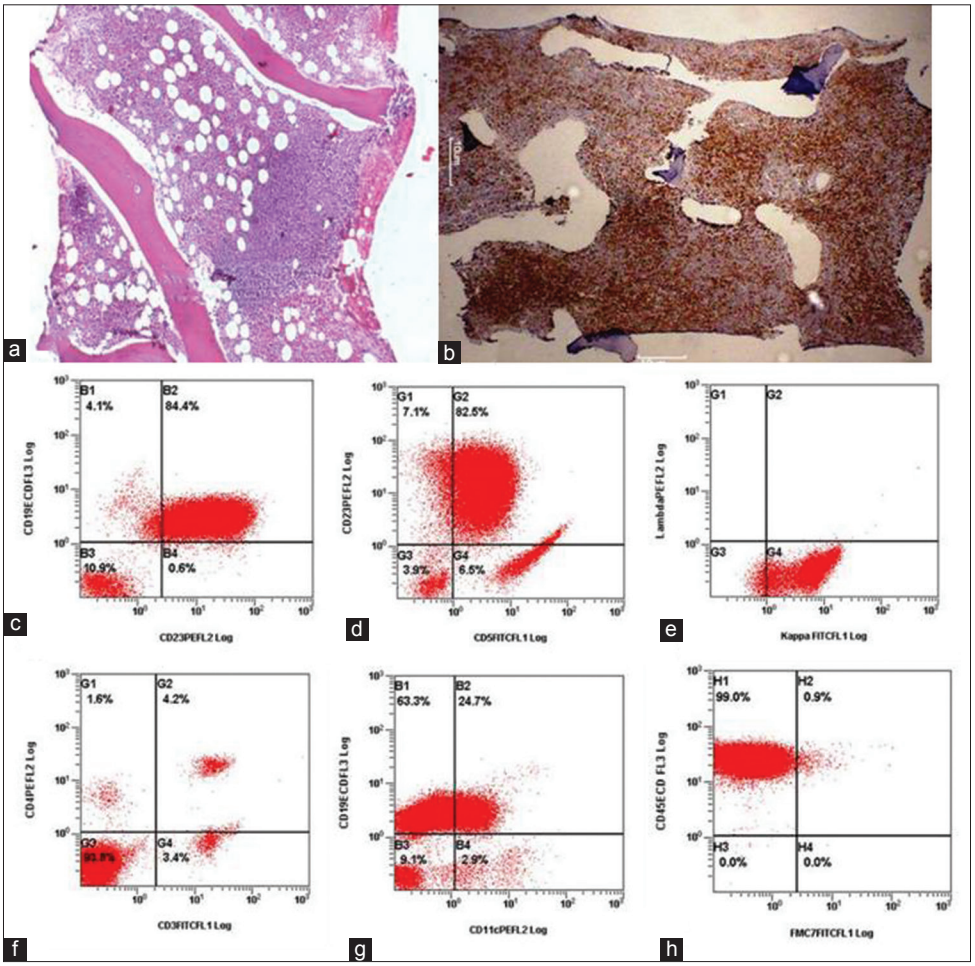

Figure 1: Bone marrow histopathology, immunohistochemistry and flow cytometric analysis in a case of chronic lymphocytic leukemia (a) nodular infiltration of bone marrow biopsy by chronic lymphocytic leukemia/small lymphocytic lymphoma, (b) CD5 and CD23 was positive in cells infiltrating the marrow, (c) 84.4% of cells in the case showed CD19 and CD23 coexpression, (d) 82.5% of cells showed CD5 and CD23 coexpression, (e) kappa and lambda plot showing kappa restriction, (f) CD3 and CD4 negativity by these cells, (g) expression of CD19 and CD23 by these cells, (h) the cells were negative for FMC7

reticulin staining, TRAP positivity of neoplastic cells on cytochemistry and positivity of neoplastic cells for CD11c, CD103, and DBA44 but negative staining for CD25 on both IHC and FCM. The patient was diagnosed as variant HCL (HCLv). A single case of SMZL was also included in the present study and it was not possible to differentiate SMZL from HCLv by morphology and routinely available markers in FCM and IHC. The 77-year-old lady diagnosed as SMZL showed a good response to therapy after being undiagnosed for a long time her repeated bone marrow aspiration showed a complete morphological remission and her spleen size returned to normal on follow-up.

Corelation between flow cytometry and immunohistochemistry

CD23 expression was noted in 20/21 CLL cases on FCM and 18/21 cases on IHC. Concordance between IHC and FCM was seen in 90% of the CLL cases for CD23 marker. Concordance between FCM and IHC for CD5 marker was noted in 100% of CLL cases and 96.6% (29/30 cases) of CLPD cases. CD20 disconcordance was found in only one case of CLPD by IHC and FCM. CD10 positivity was noted in one case of FL but was negative in the other case. Although CD10 negative, this case on morphology and complete immunophenotyping (BCL-2, CD20, and CD79b positivity) had a marrow infiltrate of FL. Lymph node biopsy in this case showed characteristic morphology of FL and IHC further confirmed the diagnosis of FL. Due to limited number of FL cases, significance of results about CD10 expression cannot be evaluated.

CD11c which is considered as a marker of HCL was found to be expressed in 4 out of 21 (19%) CLL but the intensity of CD11c expression was not as bright as was seen in HCL. Similarly, CD25 marker of HCL was found to be expressed in other types of B-CLPDs including nearly 20% of CLL. However, the expression of CD25 was brighter in HCL than in other CLPD cases.

CD19 was positive in all B-CLPDs and negative in both T-CLPDs by both FCM and IHC. CD20 was positive in 27 of 28 B-CLPD cases and negative in both T-CLPD cases by FCM but all B-CLPD cases were positive and both T-CLPD cases were negative by IHC. The concordance rates of various immunological markers on FCM and IHC is listed in Table 3 and the concordance rate was ≥ 80% for all markers. The overall concordance between FCM and IHC was 93.3%.

Table 1: Reactivity of various monoclonal antibodies on FCM in CLL and non-CLL patients

Marker score in CLL	Percentage scoring 1 in CLL (%)	Percentage scoring 0 in non-CLL (%)
CD5 positive=1	19/21 (90.5)	5/7 (71.4)
CD23 positive=1	20/21 (95.2)	3/7 (42.8)
SmIg weak positive=1	21/21 (100)	2/7 (28.5)
FMC7 negative=1	14/21 (66.6)	6/7 (85.7)
CD22 negative/weak positive=1	13/21 (61.9)	3/7 (42.8)
CD79b negative=1	18/21 (85.7)	5/7 (71.4)

CLL: Chronic lymphocytic leukemia, FCM: Flow cytometry, SmIg: Surface membrane immunoglobulin

Table 2: Comparison of Panels A, B and C in CLL and non-CLL

Type of B-CLPD	Panel A		Total	χ^2	*P*
	Score ≥4	Score <4			
CLL	18	3	21		
Non-CLL	1	6	7		
Total	19	9	28	10.26	<0.01
	Panel B				
	Score ≥4	Score <4			
CLL	20	1	21		
Non-CLL	1	6	7		
Total	21	7	28	14.2	<0.001
	Panel C				
	Score ≥3	Score <3			
CLL	18	3	21		
Non-CLL	3	4	7		
Total	21	7	28	3.3	>0.05

CLL: Chronic lymphocytic leukemia, CLPD: Chronic lymphoproliferative disorders

Table 3: Comparison of FCM and IHC in chronic lymphoproliferative disorders (*n*=30)

Marker	Number of positive cases on flow cytometry (*n*=30)	Number of positive cases on IHC (*n*=30)	Number of cases with concordant FCM and IHC (*n*=30; %)	χ^2	*P*
CD5	23	22	29 (96.6)	20.45	<0.001
CD23	24	20	26 (86.6)	11.48	<0.001
CD22	11	13	28 (93.3)	19.2	<0.001
CD10	01	01	30 (100)	6.99	<0.001
Kappa	13	17	24 (80)	9.44	<0.001
Lambda	08	09	27 (90)	13.64	<0.001
CD11c	08	06	28 (93.3)	16.20	<0.001
CD79b	20	18	26 (86.6)	12.6	<0.001
CD25	07	05	28 (93.3)	14.9	<0.001
CD3	03	03	30 (100)	19.9	<0.001
CD4	02	02	30 (100)	16.08	<0.001
CD8	01	01	30 (100)	6.99	<0.001

IHC: Immunohistochemistry, FCM: Flow cytometry

DISCUSSION

The 2008 WHO classification has emphasized that CLPD's cannot be classified on the basis of morphology alone and supplementation with immunophenotyping is indispensible, especially in the diagnosis of difficult cases.[1]

In the present study, we compared the role of FCM and IHC in the diagnosis and classification of CLPD. Thirty newly diagnosed cases of CLPD from a tertiary care hospital were evaluated by FCM on peripheral blood and/or bone marrow aspirate samples. We also assessed the morphological

patterns in bone marrow biopsy and IHC on bone marrow biopsy.

Twenty eight of 30 cases were of B-type CLPD of which CLL was the most common entity (75%). We observed expression of CD5, CD23, weak SmIg positivity and CD79b negativity were the most significant findings in the CLL group. In the non-CLL group, negativity for CD5 and positivity for FMC7 and CD79b were the most significant findings [Table 1]. These findings are comparable to observations made by Moreau *et al*. except that we did not find SmIg to be a highly expressed in CLL cases.[3] In our study, 5 of 7 non-CLL cases also showed SmIg positivity. A comparison of the three panels of monoclonal antibodies proposed by Matutes *et al*. and Moreau *et al*. for the differentiation of CLL from non-CLL was made.[3,4] We found that both Panels A and B were useful in differentiating CLL from non-CLL. The sensitivity of Panels A, B and C for CLL was 85.7%, 95.2% and 85.7%, respectively. The specificity of Panels A, B and C was 85.7%, 85.7% and 57.1%, respectively [Table 2]. The results were statistically more significant for Panel B, indicating its superiority over the other two panels. Moreau *et al*. had also found a higher accuracy of Panels B and C in comparison to Panel A.

The concordance between FCM and IHC calculated in our study was 93.3%. In a previous study by El-Sayed *et al*. in 2008 done on Egyptian patients, 88% concordance between FCM and histopathology/IHC in the diagnosis of lymphoma was reported.[5] Similar results were independently obtained by Mand'áková *et al*. and Martínez *et al*. who found a concordance between FCM and histopathology/IHC in 89% and 87.2% respectively.[6,7] Sah *et al*. compared the results between the two methods of immunophenotyping in 110 patients of B-cell lymphomas and reported a concordance rate of 88%.[8] Carulli *et al*. reported that 89.5% of 114 cases studied showed concordance between FCM and histopathological examination.[9] Naughton *et al*. in 1998 reported a lower concordance between FCM and bone marrow biopsy BMB in 273 bone marrow samples from NHL patients, where FCM detected disease in only 60% cases with bone marrow infiltration.[10] An improvement in the technique and antibodies used presently could account for the difference in the rate of concordance. Concordance rate for various immunological markers was reported between 76% and 100% by Biesemier *et al*. with 100% concordance between FCM and frozen-section immunophenotyping in CD3, CD4 and CD8 markers, which is same as that observed in this study.[11] The concordance rate for CD5, kappa and lambda was reported as 86%, 70% and 60% respectively by Biesemier *et al*. which are lower than those obtained in this study.[11]

In this study, considerable discordance was noted between IHC and FCM results in detecting kappa and lambda chain expression. Sometimes, it was difficult to interpret the Ig light chain detection by IHC because of diffuse staining in paraffin embedded blocks. Whereas in contrast, clonality assignment of the same?? By FCM was easier. Discordance was seen in 6/30 cases for kappa and 3/30 cases in lambda. Leers *et al*. had pointed out that lack of contrast between surface-immunoglobulin staining and extracellular immunoglobulin staining was a major drawback of immunohistochemical detection of monoclonality in B-cell lymphoproliferative disorders.[12] They established monoclonality in 9 out of 10 NHL cases by FCM while only 6 of 9 cases were conclusive by IHC.[12] Difficulty in determination of monoclonality by immunoglobulin light chain restriction using IHC was also noted by Abdel-Ghafar *et al*. because of the destruction of some of the antigenic epitopes by the fixation and decalcification process used for BMB specimens.[13] A similar observation had been made by Dunphy who had highlighted the difficulty arising due to weak expression of antigen in paraffin tissue because of variations in tumor preservation and fixation.[2]

We propose a two tier approach in immunophenotypic analysis of all new CLPD cases in order to derive maximum benefit from a machine (FCM) that uses costly reagents since cost can be an important limiting factor in developing countries. The screening panel comprises 9 markers including CD19, CD5, CD23, FMC7, CD10, CD20, CD3, kappa and lambda, which are important for specifying the lineage (B or T), to differentiate CLL from non-CLL group and in deciding the secondary panel. The rationale for selecting a B-cell-centric screening panel for CLPD is that majority of the cases that we found in our study were of B-CLPDs. We propose that CLPD panel should be initially meant for characterizing CLL and to differentiate it from non-CLL. Scoring system using CD5, CD23, CD22, FMC7, CD79b and SmIg is very useful in differentiating CLL from non-CLL cases. Using the 9- marker panel, a diagnosis of CLL, MCL and FL, the three most common CLPDs could be made. In a case suspected to be HCL, a secondary panel comprising of CD19, CD25, CD103, CD11c, kappa and lambda light chains, annexin 1 and DBA 44 would be required. If the T-cell markers in the screening panel are positive, T-cell CLPDs should be further evaluated using CD4, CD8, CD7.

CONCLUSION

The concordance rate between FCM on peripheral blood/bone marrow aspirate samples and IHC on bone marrow biopsy sections in our study was 93.3%. Scoring system using CD5, CD23, CD22, FMC7, CD79b and SmIg was found to be useful in differentiating CLL from other B-CLPDs. In a case of CLPD, a screening panel comprising CD19, CD5, CD23, FMC7, CD10, CD20, CD3, kappa and lambda would

be successful in the diagnosis of most of CLPDs. This can be followed by a secondary panel as required.

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Conflicts of interest
There are no conflicts of interest.

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