Original Article

Flow Cytometry Immunophenotypic Diagnosis of B-Cell Non-Hodgkin Lymphomas on Fine-Needle Aspirate of Lymph Node

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Abstract

Introduction: B-cell non-Hodgkin lymphomas (NHLs) are divided into low and high grade, typically corresponding to indolent (slow-growing) lymphomas and aggressive lymphomas, respectively. In recent years, flow cytometric immunophenotyping (FCI) has become an important tool in the diagnosis of mature lymphoid neoplasms and the determination of prognosis in selected cases. **Objective:** The aim of this study was to diagnose B-cell NHL by FCI on fine-needle aspiration (FNA) of lymph node following immunophenotypic diagnostic criteria based on the expression of CD markers. **Patients and Methods:** All samples were preliminary assessed by FNA cytology as NHL or lymphoproliferative disorder. FCI was performed with a complete panel of antibodies (CD3, CD4, CD8, CD5, CD7, CD10, CD19, CD20, CD23, CD22, CD25, CD30, CD45, CD79a, CD79b, CD95, CD56, FMC7, CD40, CD15, Kappa, Lambda, and Bcl-2) by dual-color flow cytometry. FCI data were interpreted to diagnose and subclassify NHL according to the WHO classification. Wherever possible, the diagnoses were compared with available immunohistochemistry (IHC). **Results:** During 1-year period (from March 2016 to February 2017), 31 cases of NHL were diagnosed by FCI of which 16 (51.6%) cases were B-cell NHL. Among 16 cases of B-cell lymphoma, 1 case (6.25%) was follicular lymphoma; 10 cases (62.5%) were diffuse large B-cell lymphoma (DLBCL); 2 cases (12.5%) were mantle cell lymphoma; 2 cases (12.5%) were small lymphocytic lymphoma; and 1 case (6.25%) was found to be B-cell prolymphocytic lymphoma. Monoclonal or polyclonal B cells with positive CD45, CD19, CD20, CD79a, and CD79b were found in all types. There was variation in CD5, CD23, CD10, Bcl-2, and FCM7. Identification by FCI is 40.3% higher in DLBCL than IHC. **Conclusion:** Application of FCI from FNA sample enhanced the diagnostic potential and avoiding the need for invasive surgical biopsies. Misdiagnosis can be avoided and help the physician to plan the treatment regimen accordingly.

Keywords: *B-cell non-Hodgkin lymphomas, fine needle aspirate, flow cytometry, immunophenotyping*

Introduction

The lymphomas are a heterogeneous group of disorders and account for up to 3% of all malignancies. The estimated incidence of non-Hodgkin lymphoma (NHL) is 5/100,000 (385,741 new cases), with a mortality rate of 2.5/100,000 (199,630 deaths) worldwide. NHLs are broadly classified into B-cell or T-cell lymphomas, depending on the lymphocytic lineage that gives rise to malignancy. B-cell lymphomas represent approximately 90% of NHLs whereas T-cell lymphomas approximately 10%[1] According to the WHO 2016, B-cell lymphoma subtypes are about 52. The major subtypes are as follows: (1) diffuse large B-cell Lymphoma (DLBCL), (2) follicular lymphoma (FL), (3) chronic lymphocytic leukemia (CLL), (4) mantle cell lymphoma (MCL), (5) marginal zone B-cell lymphoma, (6) Burkitt's lymphoma, (7) lymphoplasmacytic lymphoma, and (8) hairy cell leukemia. Mature B-cell neoplasms comprise 4% of new cancers each year around the world. They are more common in developed, particularly the United States, Australia, New eland, and Europe. In the United States, B-cell neoplasms, of all cancers including NHL (50,000 cases), CLL (7800 cases), and plasma cell myeloma (13,700 cases), account for over 70,000 new cases/year or 6%.[2]

NHL is relatively common in Bangladesh and contributing to 7% of total cancer reported at the Department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU) as compared to 4% in the USA.[3,4]

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B-cell lymphomas are typically divided into low and high grade, typically corresponding to indolent (slow-growing) lymphomas and aggressive lymphomas, respectively. In general, indolent lymphomas respond to treatment and can be kept under control (in remission) with long-term survival of many years, but not curable. Aggressive lymphomas usually require intensive treatments, with some having a good prospect for a permanent cure. Prognosis and treatment depend on the specific type of lymphoma as well as the stage and grade. Treatment includes radiation and chemotherapy. Early-stage indolent B-cell lymphomas can often be treated with radiation alone, with long-term nonrecurrence.[5-7] In recent years, flow cytometry has become an important tool in the diagnosis of mature lymphoid neoplasms and the determination of prognosis in selected cases. The advantages of flow cytometry are based largely on its ability to analyze, rapidly and simultaneously, multiple cell properties in a quantitative manner. Flow cytometric immunophenotyping (FCI) is a useful tool in diagnostic hematopathology. Types of specimens suitable for FCI include peripheral blood, bone marrow aspirates, core biopsies, fine-needle aspirates (FNAs), fresh tissue biopsies, and all types of body fluids.[8] Several studies have supported the usefulness of FCI in diagnosing lymphoma in fine-needle aspiration (FNA) sample as well as in the staging and follow-up of cases.[6,9,10]

FCI has become a widely used laboratory procedure for diagnosis and subtyping of lymphoma. It is an objective and quantitative diagnostic tool that allows a quick multiparametric analysis of a very large number of cells (20,000–50,000 cells per sample) which could be obtained from small tissue sample (0.1 cm^3 or even smaller).[11,12]

FCI is useful in diagnosing lymphoma under the WHO classification system, where lymphoid neoplasms are separated into distinct clinical entities based on morphology, immunophenotype, genetic abnormalities, and clinical features. The aim of this study was to diagnose B-cell NHLs by FCI on FNA of lymph node.

Patients and Methods

Flowcytometric immunophenotyping (FCI) was done on fine-needle aspirates (FNA) of lymph node diagnosed by FNA cytology (FNAC) as lymphoproliferative disorders (LPDs) from March 2016 to February 2017 at the Department of Microbiology and Immunology of BSMMU, Dhaka with approval of the Institutional Review Board of BSMMU.

Fluorescently labeled antibodies and isotype control studies

FCI was performed on three lasers, 8-color Becton Dickinson (BD) FACSverse flow cytometer. Among the three lasers (405 nm-violet lasers; 488-nm blue laser; 633-nm red laser), two lasers (blue laser and red laser) and 6-color were used in this study. The specific fluorescently labeled anti-human monoclonal antibodies used in this study were obtained from Abcam Biotechnology Company and BD. Monoclonal antibodies used for Hodgkin and NHL panel were CD45-APC-H7, CD19-PECY7, CD3-PerCpCy5.5, CD20-APC-H7, CD79a-PE, CD15-FITC, CD30-APC, CD40-PerCpCy5.5, CD95-PE, CD5-APC, CD22-PerCpCy 5.5, CD23-PE, CD79b-PerCpCy5.5, Bcl-2-APC, FMC7-FITC, CD10-APC, CD25-PerCpCy5.5, CD4-PE, CD8-FITC, CD7-FITC, CD56-APC, Kappa-FITC, and Lambda-PE. Defining 6-color FC tube was used in this study. Appropriate isotype control studies to determine background fluorescence were also used.

Sample collection

FNAs were collected from the lymph node of size >2 cm by expert pathologist. FNAC using hematoxylin and eosin (H and E) stain was made by a cytopathologist in the pathology department of BSMMU. One part of the aspirate was used to prepare smears for FNAC, and the another part of the aspirate was flushed into 500 µl phosphate buffer solution (PBS) used for flow cytometric immunophenotyping.

Flow cytometry analysis and interpretation

FNA samples were processed as soon as possible mostly within 2–3 h of collection for better result. A "stain and then lyse/wash" technique was used for processing of samples according to BD FACS Verse™ Manual 2013.

For identification of surface markers

100 µl of the sample was taken in each tube to ensure approximate concentration of 10/ml. 2 ml BD FACS lysing solution was taken in each tube, vortexed, and incubated in dark at room temperature for 10–20 min. Then, the cells were spuned at 200–300 g for 3–5 min, and supernatant fluid was discarded. Cells were washed with sheath fluid, vortexed, spuned and supernatant was discarded. Pretitrated volume of fluorochrome antibody was added in each tube, vortexed, incubated in dark at room temperature for 10–15 min, 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 the identification of intracellular markers pretreated 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. 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 were done using BD FAC suite software™ version 1.0.3 (BD Biosciences). The diagnostic criteria were used for FCI of lymphoma according to revised the WHO classification of tumors of hematopoietic and lymphoid tissues (2016).[13]

Results

Among 31 NHL cases identified by FCI, 16 (51.6%) cases of B-cell type of NHL were identified during 1-year period (from March 2016 to February 2017). All cases were screened for atypical lymphocytes by FNAC which suggested the cases as LPD or NHL. The age range was between 22 and 80 years with 13 males and 3 females.

Subtypes of B-cell lymphoma by FCI are depicted in Table 1 where among 16 cases of B-cell lymphoma, 1 case (6.25%) was FL; 10 cases (62.5%) were DLBCL; 2 cases (12.5%) were MCL; 2 cases (12.5%) were SLL; and 1 case (6.25%) was found to be B-cell prolymphocytic lymphoma (BPLL).

FCI criteria for the diagnosis of B-cell type of NHL has been shown in Table 2 where monoclonal or polyclonal B cells with positive CD45, CD19, CD20, CD79a, and CD79b were found in all types. There was variation in CD5, CD23, CD10, Bcl-2, and FCM7. Small lymphocytic lymphoma (SLL) showed a positive reaction to CD45, CD19, CD5, CD23, CD79b, Bcl-2, and negative reaction to CD10 and FMC7 with lambda light chain restriction [Figure 1]. DLBCL cases were negative for CD5 and CD23 but positive for CD45, CD19, CD20, CD 22, CD79b, FMC7, Bcl-2 with light chain restriction [Figure 2].

Table 1: Subtypes of B-cell lymphoma identified by flowcytometric immunophenotyping (*n*=16)

Subtypes	*n* (%)
FL	1 (6.25)
DLBCL	10 (62.5)
MCL	2 (12.5)
SLL	2 (12.5)
BPLL	1 (6.25)

FL: Follicular lymphoma, DLBCL: Diffuse large B-cell lymphoma, MCL: Mantle cell lymphoma, SLL: Small lymphocytic lymphoma, BPLL: B-cell prolymphocytic lymphoma

The correlation between FCI and immunohistochemistry (IHC) was done on 17 available IHC results. FCI findings showed that 8 (47.1%) cases were B-cell type NHL whereas on IHC, 9 (52.9%) cases were B-cell type NHL. Identification by FCI is 40.3% higher in DLBCL than IHC [Figure 3].

Discussion

Flow cytometry analysis on FNA of lymph node could reveal 51.6% of cases of B-cell NHL. In this study, a study carried out in India reported 65.5% B-cell lymphomas which has a close similarity with ours.[14] B-cell type of lymphoma cases is diagnosed and categorized by expression of CD5. CLL/SLL and MCL are diagnosed by expression of CD5 on B-cell while other B-cell type of lymphoma are diagnosed by the absence of CD5 on B-cell. Among the 16 B-cell type NHL, 10 (62.5%) cases were DLBCL following criteria set by many cases.[15,16] Among the 10 cases of DLBCL, 8 (80%) cases were negative for CD5 and CD23 but positive for CD45, CD19, CD20, CD79a, CD79b, FMC7 with light chain restriction with the exception of 2 (20%) cases where CD5 was positive. One study described that around 10% of DLBCL express CD5 which may be seen in transformed CLL or MCL.[16] The expression of CD5 marker in DLBCL has also been reported by other studies.[17]

As the large proportion of the lymphoma cases was of B-cell origin, among which majority was DLBCL 10 (62.5%), followed by insignificant number of 6%–12% were of other varieties namely FL, SLL, MCL, and BPLL, respectively. Findings of this study are consistent with other studies.[18] A study from Italy is not in agreement with ours as 38% of their cases were FL followed by DLBCL (23.9%) while 7%–8% were MCL and SLL.[19] A study in India reported less number of DLBCL cases (25%) but their findings was almost similar to our study regarding FL, SLL, and MCL cases which was 6%–15%.[14] This difference from our findings regarding DLBCL could not be explained, although ethnically Indian population is diversed which is not in our cases. Very much similar findings has been reported by others.[20]

One (6.5%) case of FL showed positive reaction to CD45, CD19, CD20, CD79a CD79b, FMC7, CD10 and negative to CD5, CD23 without light chain restriction in our study.

Table 2: Flow cytometric immunophenotyping criteria for diagnosis of B-cell type of nonhodgkin lymphomas

Diagnosis	CD45	CD19	CD20	CD5	CD23	CsD10	CD79a	CD79b	FMC7	K/L	BCL2
FL	+++	+++	+++	−	−	+	+	++	++	Clonal/poly	−
SLL	+++	+++	+++	++	++	−	+/-	++	−	Clonal/alter	−
DLBCL	+++	+++	+++	−	−	-/+	++	+++	+	Clonal/poly	+/−
MCL	+++	+++	+++	+	−	−	+	++	−	Clonal/poly	++
B-PLL	+++	+++	+++	−	−	−	+	++	+	Clonal	+

FL: Follicular lymphoma, SLL: Small lymphocytic lymphoma, DLBCL; Diffuse large B-cell lymphoma, MCL: Mantle cell lymphoma, BPLL: B-cell prolymphocytic lymphoma. Clonal: Monoclonal, Poly: Polyclonal, Alter: Altered Kappa/Lamda ratio but no light chain restriction. +++: Strong intensity, ++: Moderate intensity, +: Dim intensity, −: Negative intensity

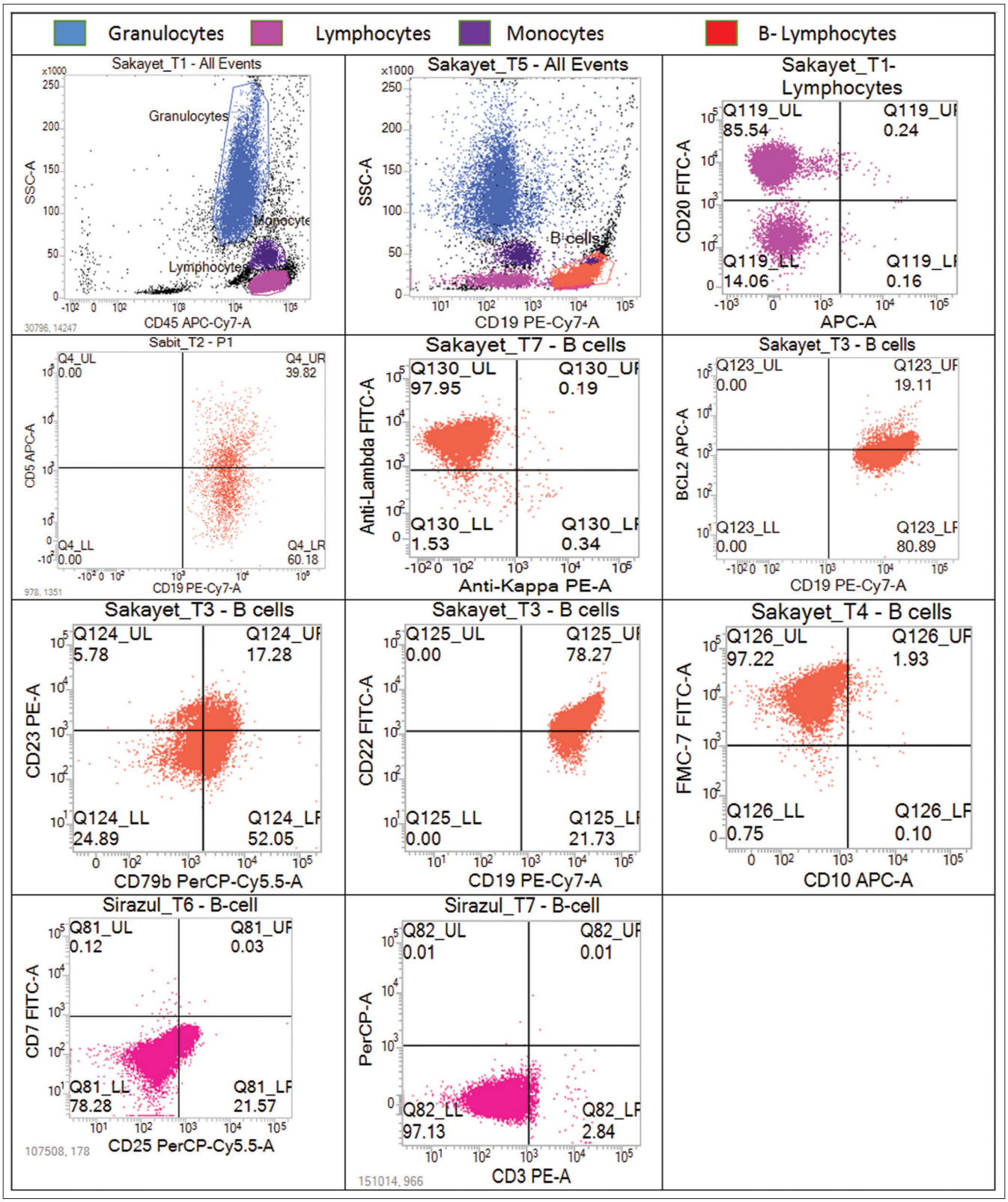

Figure 1: Flow cytometric immunophenotypic findings in a patient with small lymphocytic lymphoma

Similar type of expression of CD markers also observed in several studies.[16,17,21,22] In this study, 2 (12.5%) cases of SLL showed positive reaction to CD45, CD19, CD5, CD23, CD79a, CD79b and negative reaction to CD10 and FMC7. One case of SLL had light chain restriction but other cases had altered light chain. Several studies observed almost similar expression of CD markers in SLL.[14,16,18,21-23]

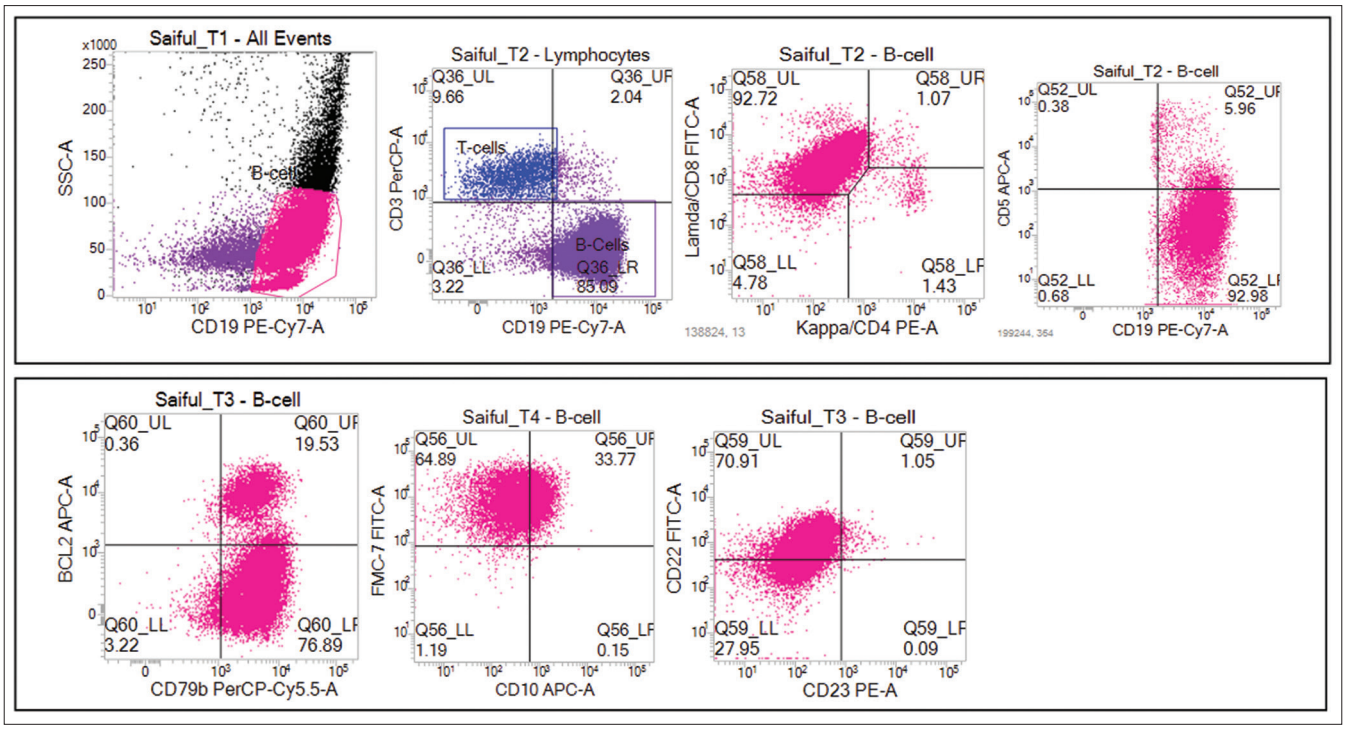

Figure 2: Flow cytometric immunophenotypic findings in a patient with diffuse large B-cell lymphoma

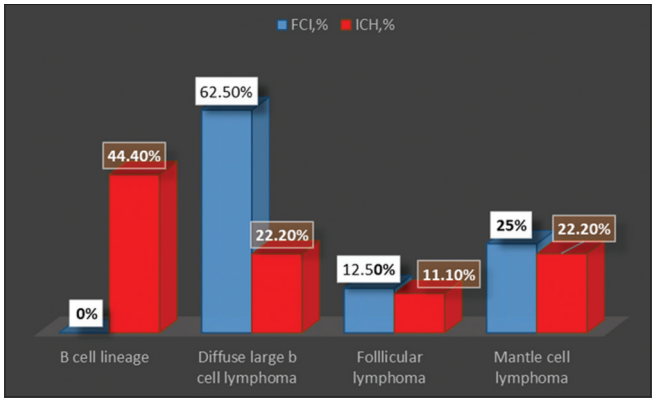

Figure 3: Correlation between flow cytometric immunophenotyping and immunohistochemistry results (*n* = 17)

In this study, 2 (12.5%) cases were MCL which showed positive reaction to CD45, CD19, CD5, BCL2 and negative reaction to CD10, CD23. Light chain restriction was observed in one case. Several studies showed similar type of CD markers expression in MCL.[14,16,21,22]

According to diagnostic criteria of Parker *et al*.,[16] 1 (6.25%) case was diagnosed as BPLL which showed a positive reaction to CD45, CD19, CD20, CD79a, FMC7, BCL2 and negative reaction to CD5, CD23, CD10 with polyclonal B-cells.

Light chain restriction is a criterion of B-cell lymphoma which is 5 (62.5%) with lamda and 3 (37.5%) with Kappa chain restriction. The absence of light chain restriction was 2 (20%) and presence with polyclonality. Other studies are of the view that light chain restriction is not mandatory in a few number of cases of B-cell NH.[20,24]

In this study, of 16 cases of B-cell type NHL, 5 (31.2%) cases had no light chain restriction but strong positive reaction to CD19 and CD20. Among these 5 cases, 1 (20%) case was FL, 1 (20%) case was MCL, 2 (40%) cases were DLBCL, but 1 (20%) case was SLL which had altered light chain restriction. A study revealed NHL cases with no light chain immunoglobulin expression and they believed if these cases were evaluated regarding light chain immunoglobulin they could be monoclonal and thus considered the cases as NHL.[25] Another study showed light chain restriction in 75% of B-cell NHL and other 25% cases did not, but considered them as B-cell NHL due to excessive expression of CD20.[20]

Therapeutic response has been reported to be associated with the presence or absence of CD10 and BCL2 expression on B cell.[26] Our study revealed that, out of 10 DLBCL cases, 2 (20%) cases were both CD10 and BCL2 positive which seems to have worse prognosis. We have also found expression of CD10 in 5 (50%) cases but 5 (50%) cases did not, while 6 (60%) cases of DLBCL were BCL2 negative and 4 (40%) were BCL2 positive, whereas 5 (50%) cases of DLBCL were negative for CD23 and CD10. A study carried out by Craig and Foon[17] revealed such expression of CD23 and CD10 in DLBCL. The identification of BCL2 before chemotherapy is important as BCL2 positivity indicates worse prognosis as they arise from follicular center of lymph node.[27]

Conclusion

Thus, this study implicates that the diagnosis of B-cell NHL from FNAs of lymph node by FCI enhanced the diagnostic potential and avoiding the need for invasive surgical biopsies. Misdiagnosis can be avoided and help the physician to plan the treatment regimen accordingly.

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Conflicts of interest

There are no conflicts of interest.

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