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Flow cytometric immunophenotyping including Bcl-2 detection on fine needle aspirates of lymph node in the diagnosis of Diffuse Large B-Cell Lymphoma

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Abstract
Diffuse large B-cell lymphoma (DLBCL) is a fast growing and aggressive high grade non-Hodgkin lymphoma (HG-NHL) that spreads quickly, present with lymphadenopathy or enlarged nodes in mediastinum, mesenteric region or peritoneum, from where tissue excision and biopsy with histopathology is not possible. The aim of this study was to diagnose DLBCL by Flow cytometric immunophenotyping (FCI) on fine needle aspirate (FNA) of lymphnode following immunophenotypic diagnostic criteria based on expression of CD markers. All samples were preliminary assessed by fine needle aspiration cytology (FNAC) as NHL or lymph proliferative disorder (LPD). 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 flowcytometry. FCI data were interpreted to diagnose and sub classify NHL according to WHO classification. Wherever possible the diagnoses were compared with available immunohistochemistry (IHC) and histopathology reports. During one year period (from February 2016 to March 2017)10 cases of DLBCL were identified by FCI. Out of 10 DLBCL cases, 8 histopathology and 6 1HC reports were available of which 6 DLBCL cases showed 100% (6/6) concordance with combined histopathology and IHC and 100% concordance with IHC alone; 75% (6/8) concordance and 25% (2/8) discordance with histopathology alone. Ig light chain was detected in 8 (80%) DLBCL cases and 40% (4/10) DLBCL cases showed Bcl-2 expression. Diagnosis of DLBCL by FCI on FNA of Lymph node can be of great help as in most cases of DLBCL biopsy of histopathology is not possible. Detection of Bcl-2 expression can help to assess the prognosis of the disease and resistance to chemotherapy.

Keywords: *Flow ytometry, Immunophenotyping, Cytology, DLBCL, HG-NHL, Bcl-2.*

Introduction

Diffuse large B-Cell Lymphoma (DLBCL) is the most common type high grade non Hodgkin lymphoma (HG-NHL) which accounts for 37% of mature B-Cell neoplasms.[1] Lymphnodes orextranodal sites such as gastrointestinal tract, testes, thyroid, skin, breast, bone, brain or essentially any organ are the sites for DLBCL. According to WHO 2016 classification there are several subtypes of DLBCL but most common are

mediastinal large B-Cell lymphoma, Plasmablastic lymphoma, T-Cell/histiocyte-rich large B-Cell lymphoma, ALK positive DLBCL, intravascular large B-Cell lymphoma, Primary effusion lymphoma.[2] DLBCL is a fast growing and aggressive HG-NHL that spreads quickly but has a better response to chemotherapy than that of low grade non- Hodgkin lymphoma. Aggressiveness of DLBCL is related with expression of Bcl-2protein which is detected in >50% of DLBCLs and~ 75% of High grade B-Cell lymphomas (HGBLs) but not expressed in B-Cell lymphomas or normal Germinal Centre B-Cell (GCB).[3,4]

As Bcl-2 inhibits apoptosis, It accelerates lymphoma progression and induce resistance to chemotherapy.[5,6]

Most patients of DLBCL present with lymphadenopathy or enlarged nodes in madiastinum, the mesenteric region or the peritoneum. Tissue excision and biopsy with histopathology is gold standard in diagnosis of lymphoma but open excision and biopsy is not possible in the aforementioned sites.[7] The use of flow cytometric immunophenotyping (FCI) on fine needle aspirate (FNA) of lymph node to diagnose DLBCL can overcome this limitation and thus is a useful tool for lymphoma diagnosis.

Flow cytometric immunophenotyping (FCI) is useful tool in diagnostic haematopathology. It is a rapid and sensitive method to detect 2 or more antigens on the same cell.[8,9] Several studies have supported the usefulness of FCI in diagnosing Lymphoma on Fine needle aspirate (FNA) sample as well as in staging and follow up of cases.[10,11,12]

FCI evaluates several antigens on one cell, give quantitative results and can detect small abnormal cell populations against a reactive back ground. Further, current techniques allow detection of intracytoplasmic antigens, thus closing the gap between FCI and immunocytochemistry (IC). These features significantly improve the diagnostic sensitivity and therefore are particularly useful in lymphoma diagnostics.[13]

Precise diagnosis is the corner stone for selection of proper treatment plan and long term survival of most cases of lymphoma. Thus it is advantageous to use FCI simultaneously with regular cytomorphological and histopathological examination to reach an exact diagnosis. The aim of this study is to diagnose DLBCL and to compare FCI result with histopathology which is gold standard, so that it can be applied on FNA for routine use. Bcl-2 marker was included to see its expression which helps to assess the prognosis of the disease and resistance to chemotherapy.

Materials and Methods

Flow cytometric immunophenotyping (FCI) was done on fine needle aspirates (FNA) of lymphnode diagnosed by fine needle aspiration cytology (FNAC) as lymphoproliferative disorders (LPD) or non-Hodgkin lymphoma (NHL) during the period from march 2016 to February 2017 at the department of Microbiology and Immunology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka with approval of the institutional review board (IRB) of BSMMU.

Fluorescently Labeled Antibodies and Isotype control studies

FCI was performed on 3 lasers, 8-color Becton Dickinson FACS verse flow cytometer. Among the 3 lasers (405nm-violet laser; 488-nm blue laser; 633-nm red laser) 2 lasers (Blue laser and red laser) and 6-color was used in this study. The specific fluorescently labeled anti-human monoclonal antibodies used in this study were obtained from Abcam Biotechnology Company and Becton Dickinson (BD). Monoclonal Antibodies used for Hodgkin and Non-Hodgkin lymphoma 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, Lambda-PE. Defining 6-color FC tube was used in this study.

Appropriateisotype control studies to determine background fluorescence were also used.

Sample Collection

Fine needle aspirates were collected from the lymph node of size >2 cm by expert pathologist.[14] Fine needle aspiration cytology (FNAC) using Haematoxylin and Eosin (H&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 other part of the aspirate was flushed in to 500ùl phosphate buffer solution (PBS) used for flow cytometric immunophenotyping.

Flow cytometry analysis and interpretation:

Fine needle aspirate samples were processed as soon as possible mostly within 2-3 hours 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

100ùl of 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 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 in to 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 BD FAC suite software version 1.0.3. The diagnostic criteria were used for flow cytometric immunophenotyping of lymphoma according to revised WHO classification of tumors of hematopoietic and lymphoid tissues (2016).[2]

Results

Ten cases of DLBCL were identified by FCI during one year period (from February 2016 to March 2017). All cases were screened for atypical lymphocytes by FNAC which suggested the cases as lymphoproliferative disorder (LPD) or non-Hodgkin lymphoma (NHL).The Age range was between 22 years to 80 years with 8 male and 2 female.

The flow cytometry findings are depicted in Table 1 and Figure 1.On FCI, 8 out of 10 (80%) DLBCL cases did not express CD5 but 2(20%) cases were CD5 positive. These cases also did not express CD23.All cases of DLBCL strongly expressed CD45, CD19, CD20, CD22, CD79a, CD79b but expression of FMC7 was moderate (Figure 1). Ig light chain restriction was detected in 8(80%) DLBCL cases but 2 cases did not show monoclonality. In DLBCL we noted a heterogenous Bcl-2 expression with 40% expressing Bcl-2 which is an antiapoptic marker with poor prognosis. CD10 expression was 50% in DLBCL cases.

Histopathology result in 8 cases and immunohistochemistry (IHC) results in 6 cases were available among 10 cases of DLBCL diagnosed on FCI and FNAC. Six cases showed 100% (6/6) concordance with combined histopathology and IHC and 100% with IHC alone, also 75%(6/8) concordance with histopathology alone (Table 2).

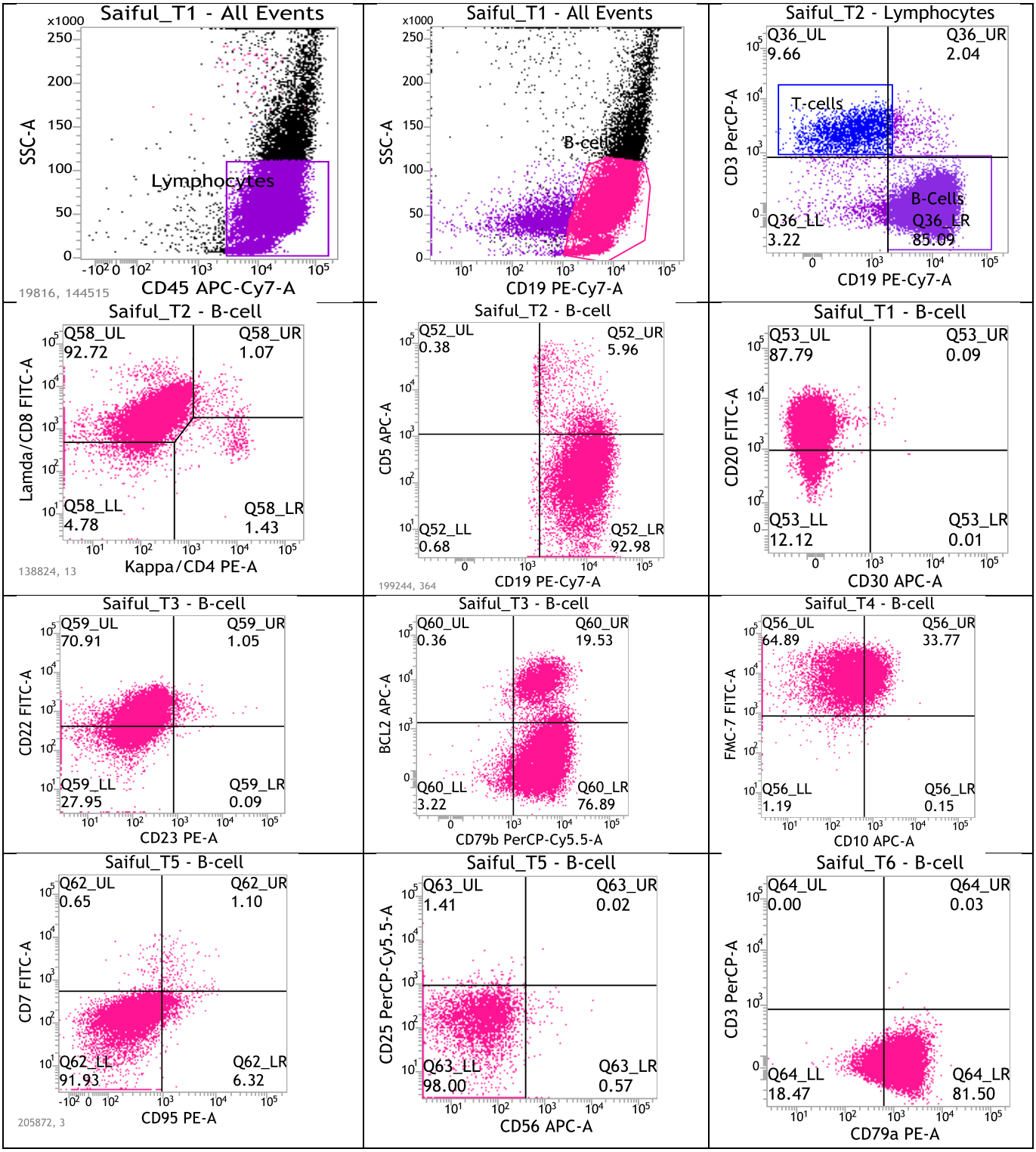

Figure -1: Flow cytometric immunophenotypic findings in a patient with DLBCL

Table-1: Flow cytometry Immunophenotypic findings of Diffuse large B-cell lymphoma

Case no.	CD45	CD19	CD20	CD5	CD23	CD10	CD79a	CD79b	FMC7	K/L	BCL2
1	+++	+++	+++	_	_	++	++	+++	+++	K	++
2	+++	+++	+++	_	_	++	+++	++	++	L	++
3	+++	+++	+++	_	_	_	+++	+++	++	L	_
4	+++	+++	+++	_	_	-_	++	+++	++	K	_
5	+++	+++	+++	+	_	_	+++	+++	++	K	_
6	+++	+++	++	_	_	_	+++	++	++	Poly	++
7	+++	+++	+++	+	_	_	+++	+++	++	L	_
8	+++	+++	+++	_	+	++	++	+++	+++	L	_
9	+++	+++	++	_	_	++	+++	++	++	L	_
10	+++	+++	+++	_	+	++	++	+++	++	Poly	++

Note: K: Kappa light chain, L: Lambda light chain, Poly: Polyclonal
Strong intensity: +++, Moderate intensity: ++, Dim intensity: +, Negative intensity

Table-2: FCI versus available histopathology and IHC diagnosis of DLBCL

Case No.	FCI Diagnosis	Histopathology	IHC
01	DLBCL	NA	NA
02	DLBCL	NA	NA
03	DLBCL	PTCL	NA
04	DLBCL	DLBCL	DLBCL
05	DLBCL	LPD	NA
06	DLBCL	DLBCL	DLBCL
07	DLBCL	DLBCL	DLBCL
08	DLBCL	DLBCL	DLBCL
09	DLBCL	DLBCL	DLBCL
10	DLBCL	DLBCL	DLBCL

DLBCL= Diffuse large B-Cell lymphoma, PTCL = Peripheral T-Cell lymphoma, FNAC= Fine needle aspiration cytology, FCI=Flow cytometry immunophenotyping, IHC=Immunohistochemistry, LPD= Lymphoproliferative disorder, NA= Not Available

Discussion

Expression of CD^5 is vital for diagnosis and categorization of B cell lymphomas. Small lymphocytic lymphoma (SLL) and Mantle cell lymphoma (MCL)express CD5 on B-cell while other B-cell type of lymphoma do not express CD5 on B cell. In this study 10 diffuse large B-cell lymphoma (DLBCL) cases were diagnosed following immunophenotypic criteria set by Mcpherson and Pincus[15] and Parker et al.[16] Accordingly, immunophenotypic diagnostic criteria of DLBCL is absence of CD5 and CD23 with strong expression of FMC7, CD45, CD19, CD20, CD22, CD79a, CD79b and light chain restriction either kappa or lambda. Among the 10 cases of DLBCL, 8(80%) cases were negative for CD5 and CD23; strong expression of CD45, CD19, CD20, CD79a, CD79b, FMC7 with light chain restriction with the exception of 2(20%) cases where CD5 was positive. Parker et al.[16] described that around 10% of DLBCL express CD5 which may be seen in transformed CLL or mantle cell lymphoma. Expression of CD5 marker in DLBCL has also been reported by another study in USA.[17]

Light chain restriction is a criteria of B-cell lymphoma which was 5(50%) with lambda and 3(30%) with Kappa chain restriction in this study. Absence of light chain restriction was 20% (2/10). Several other studies are of the view that light chain restriction is not mandatory.[18,19]

Another study showed no light chain immunoglobulin expression and considered as NHL by the presence of other marker.[20] A study in Kuwait showed light chain restriction in 75% of B-cell NHL, but the rest 25%withoutlight chain restriction were considered NHL due to significant expression of CD20.[18]

Therapeutic response has been reported to be associated with the presence or absence of CD10 and Bcl-2 expression on B-cell.[21]In this study out of 10 DLBCL cases, 2(20%) cases were both

CD10 and Bcl-2 positive which indicate a worse prognosis. Identification of CD markers like CD10, Bcl-2, CD23 is important because of the poor response of these cases to chemotherapy as they arise from the follicular center of lymph node.[22]In this study, 5(50%) cases expressed CD10 and 5(50%) cases did not while 6(60%) cases of DLBCL were BCL2 negative and 4(40%) were BCL2 positive; while 5(50%) cases of DLBCL were negative for CD23 and CD10. Other studies reported similar findings regarding expression of CD23 and CD10.[17]

Diagnosis of Lymphoma by conventional histopathology and IHC from tissue biopsy does not provide all information regarding the treatment outcome. Although there has been 100% concordance between IHC and FCI in this study, FCI has an edge over IHC as it can detect prognostic marker like Bcl-2 and other markers which determine the outcome of the chemotherapy. Although Bcl-2 is a prognostic marker its expression may be down regulated as described by Lai et al.[23]In our Bcl-2 negative cases this down regulation cannot be ruled out. So Bcl-2 may not be a reliable marker in all cases and each case need to be evaluated on the basis of other marker also.

Bcl-2 detection on a single B cell is usually associated with concomitant detection of other B cell markers. So that malignant cell can be differentiated from normal germinal center cell, as in this study dual staining was applied with monoclonal antibodies to Bcl-2 and CD79b, while others like cornfield et al.[24]used Bcl-2 with CD20 and cook et al.[25]used 3 colors FCM panel of CD10, CD20 and Bcl-2 monoclonal antibodies.

Flowcytometric analysis of lymphoma cases is changing with the invent of new technologies and corresponding dye tag markers as well as availability of monoclonal antibodies. So, studies need to be carefully evaluated considering above factors.

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