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Hodgkin and Non-Hodgkin Lymphoma: Flowcytometric Immunophenotyping on Fine Needle Aspirate of Lymph Node

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ABSTRACT

Introduction: *Hodgkin and Non-Hodgkin lymphoma (NHL) differ substantially in response to therapy and course. So accurate differentiation is important for therapeutic decision.*

Objective: *The aim of this study was to evaluate the application of flow cytometry in diagnosis of Hodgkin and Non--Hodgkin lymphoma on fine needle aspirate (FNA) of lymph node by following immunophenotypic diagnostic criteria based on expression of CD markers.*

Method: *Fine needle aspiration cytology (FNAC) was done on 40 clinically suspected lymphoma cases. If atypical lymphocytes were present FCI was performed with a complete panel of monoclonal antibodies (CD3,CD4,CD8,CD5,CD7,CD10,CD19,CD20,CD22,CD23,CD25,CD30,CD45,CD79a,CD79b,CD95,FMC7,CD40,CD15,CD56, Kappa, Lambda and Bcl-2) by dual flow color cytometry. FCI data were interpreted to diagnose lymphoma according to WHO classification. Wherever possible the diagnosis was compared with available histopathology and immunohistochemistry (IHC) reports.*

Result: *Out of 40 cases, 32 (80%) cases were diagnosed and characterized as lymphoma. Among 32 cases, 31 (96.9%) cases were Non-Hodgkin lymphoma (NHL) and 1 (3.1%) case was Hodgkin lymphoma (HL). Among 29 histopathology reports available, comparison between FCI and histopathology showed concordance (both complete and partial) in 13 (44.8%) cases and discordance in 16 (55.2%) cases. Among 17 immunohistochemistry (IHC) reports available, comparison between FCI and IHC showed concordance (both complete and partial) in 12 (70.6%) cases and discordance in 5 (29.4%) cases.*

Conclusion: *FCI from FNA sample can enhance the diagnostic potential and avoid the need for invasive surgical biopsies. Moreover, it can diagnose more Non-Hodgkin lymphoma than Hodgkin lymphoma.*

Keywords

Flow cytometry, Immunophenotyping, Cytology, Non-Hodgkin lymphoma, Hodgkin lymphoma.

Introduction

The lymphomas are a heterogenous group of disorders and accounts for up to 3% of all malignancies. Lymphoma, a cancer of the lymphocytes, occurs when cells grow abnormally and out of control. Lymphoma usually begins in a lymph node. But it can also begin in the stomach, intestine, skin or any other organ [1]. Classification of lymphoma is a little bit complex. But it can be classified into 2 broad headings as Hodgkin and Non-Hodgkin lymphoma. The world Health Organization recognizes three major categories of lymphoid neoplasms- B-cell neoplasms; T-cell and natural killer (NK) cell neoplasms; Hodgkin lymphoma [2]. Non-Hodgkin lymphoma is five times more common than Hodgkin lymphoma. In Bangladesh, prevalence of lymphoma is 20.8% of all hematological malignancies in which Non-Hodgkin lymphoma 16.9% with median age 48 years and Hodgkin lymphoma 3.9% with median age 36 years [3]. Traditionally, the

technique of choice for diagnosis of lymphoma was based on the histopathology study of paraffin embedded tissue. Currently the use of immunophenotyping techniques for detecting cell specific antigens is essential in classifying tumors, identifying prognostic factors, and identifying targets for therapy [4].

Tissue excision and biopsy with histopathology is the gold standard in diagnosis of lymphoma. In some instances, open excision and biopsy is not possible, e.g. mediastinal lymphadenopathy, retroperitoneal lymphadenopathy or ocular lymphadenopathy. Most laboratories use IHC for lymphoma diagnosis but it has many limitations. For IHC all CD markers are not available (such as; CD13, CD14, CD19, CD33 etc), it is time consuming and only biopsy samples can be examined [5]. Immunophenotyping can be done in several ways and one of the recent ways is Flowcytometry. Flow cytometric immunophenotyping (FCI) is a useful tool in diagnostic hematopathology. Types of specimens suitable for FCI include peripheral blood, bone-marrow (BM) aspirates, and core biopsies, fine needle aspirates (FNAs), fresh tissue biopsies and all types of body fluids [6]. FCI has become a widely used laboratory procedure for diagnosis and subtyping of lymphoma. It is an objective and quantitative diagnostic tool that allows 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 cm3 or even smaller). Meanwhile analysis of such small sample is facilitated by applying dual and triple markers that permit in a single experiment and the detection of expression of combination of 2 or 3 antigens respectively on the same cell [7,8].

In this technique, distinct cell populations are defined by their size (forward light scatter) and granularity (side light scatter), weakly expressed surface antigens may be detected, two simultaneous hematological malignancies may be detected within the same tissue [9]. FCI can detect abnormal cell population against reactive background. Further, current techniques allow detection of intra cytoplasmic antigens. These features significantly improve the diagnostic sensitivity in lymphoma diagnosis [9]. The incidence of lymphoma is increasing and they cause significant morbidity and mortality. Appropriate diagnosis of lymphoma is essential for introduction of early and proper treatment regime to reduce mortality and in some cases for complete cure of the patient. So, this study was conducted to diagnose and subtyping of lymphoma from clinically suspected cases of all age group by flow cytometric immunophenotyping. The main goal is to identify the best possible application of FCI in diagnosis of Hodgkin and non-Hodgkin lymphoma on fine needle aspirate of lymph node.

Materials and Methods

Patients and methods

Flowcytometric immunophenotyping (FCI) was done on fine needle aspirates (FNA) of lymph node diagnosed by fine needle aspiration cytology (FNAC) as lymphoproliferative disorders (LPD) 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.

A total 40 clinically suspected cases of Lymphoma were enrolled in this study after taking informed written consent from the patients, who were attending in the Department of Pathology, BSMMU. All data were recorded in the predesigned data sheet. Reports of cytomorphology, histopathology and immunohistochemistry were collected. Reports of bone marrow study, peripheral blood and complete blood count (CBC) were collected as supportive documents.

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. Appropriate isotype 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. 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 flowcytometric 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 fluorochrome 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 pre calibrated 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) [10].

Results

Out of 40 clinically suspected lymphoma cases, most cases, 19 (59.4%), were belonged to the age group of 30-59 years. The age distribution in study cases were <30 years 7 (21.8%), 30-59 years 19 (59.4%), and >60 years 6 (18.8%) (Figure 1).

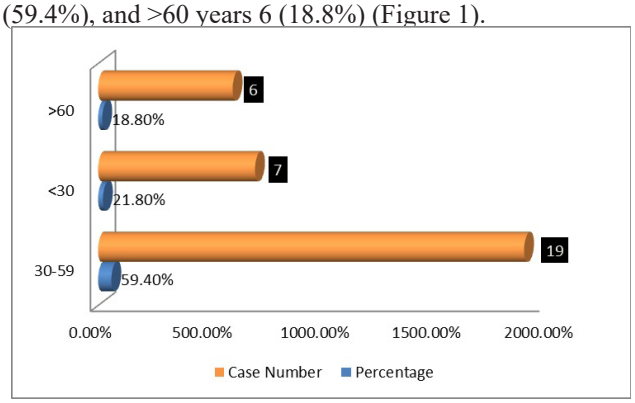

Figure 1: Distribution of study population by age.

Out of 40 cases, 32 (80%) cases were diagnosed and characterized as lymphoma, 1 (2.5%) case was diagnosed as reactive hyperplasia (RH) and 7 (17.5 %) cases were diagnosed as not consistent with lymphoma by FCI. Among the 32 cases diagnosed as lymphoma, 31 (96.9%) cases were diagnosed as Non-Hodgkin lymphoma (NHL) and 1 (3.1%) case was diagnosed as Hodgkin lymphoma (HL) (Table 1).

Groups	Number	Percentage (%)	Lymphoma Subtype	Number	Percentage (%)
Lymphoma	32	80	Hodgkin lymphoma Non-Hodgkin lymphoma	1 31	3.1 96.9
Reactive Hyperplasia (RH)	1	2.5			
Not consistent with lymphoma*	7	17.5			

Table 1: Flow cytometric analysis of fine needle aspirates (FNA) of lymph node (n=40).

*Where 7 cases could not be diagnosed by FCI as lymphoma and were reported as not consistent with lymphoma. Among these 7 cases, 2 cases were under chemotherapy; 1 case was diagnosed as sarcoma and 1 case was diagnosed as metastatic adenocarcinoma by histopathology; 1 case was diagnosed as Lymphoproliferative disorder, 1 case as Chronic non -specific lymphadenitis and 1 case was suggestive of Hodgkin lymphoma by cytomorphology.

Figure 2 shows typing of NHL cases which were diagnosed by Flow-cytometric immunophenotyping (FCI). Where among 31 NHL cases, 51.6% were B-cell type, 41.9% were T-cell type, and 6.5% cases were diagnosed as NK cell type of NHL.

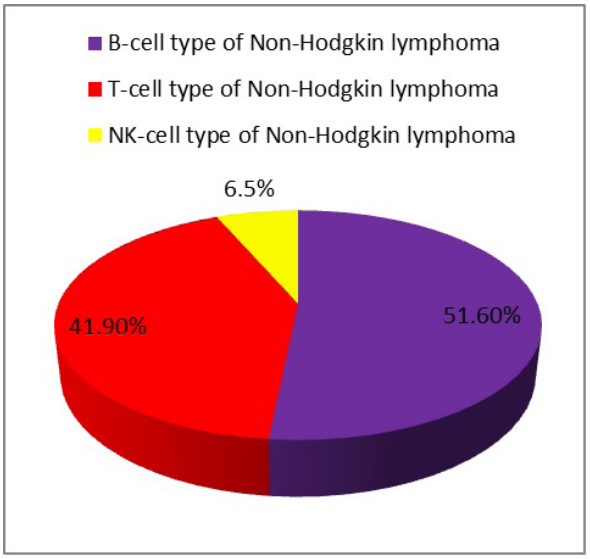

Figure 2: Types of Non-Hodgkin lymphoma (NHL) identified by Flow cytometric immunophenotyping (n=31).

Among 29 cases, 1 case was HL by FCI, whereas 7 case were HL by histopathology; 23 cases were NHL by FCI but 15 cases were NHL on histopathology (Figure 3).

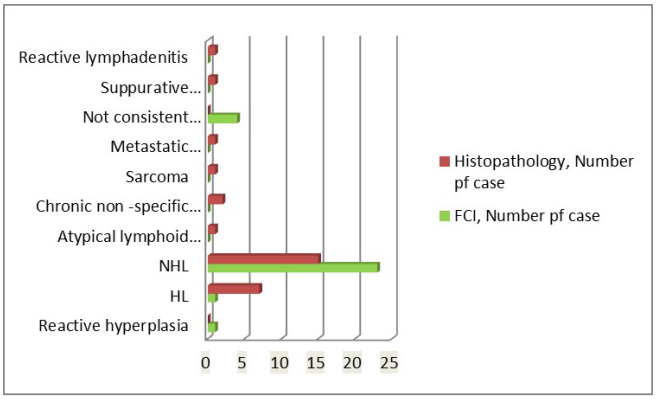

Figure 3: Correlation between FCI and histopathology (n=29).

Out of 40 cases, only 17 IHC reports were available. FCI findings showed, 8 (47.1%) cases were B-cell type NHL, 6 (35.2%) cases were T-cell type NHL, 1 (5.9%) case NK cell type (Table 2).

Figure 4 shows concordance and discordance between results

of Flowcytometry and Histopathology where Out of 29 cases, complete concordance and partial concordance was found in 17.2% and 27.6% cases respectively. Discordant result was seen in 55.2% cases.

Groups	FCI	IHC
Hodgkin lymphoma	0	4 (23.5%)
Non-Hodgkin lymphoma (B-cell type)	8 (47.1%)	9 (52.9%)
Non-Hodgkin lymphoma (T-cell type)	6 (35.2%)	2 (11.8%)
NK cell type of NHL	1 (5.9%)	0
Reactive changes	0	1 (5.9%)
Myeloid Sarcoma	0	1 (5.9%)
Not consistent with lymphoma	2 (11.8%)	0

Table 2: Correlation between findings of flowcytometric immunophenotyping (FCI) and immunohistochemistry (IHC) (n=17).

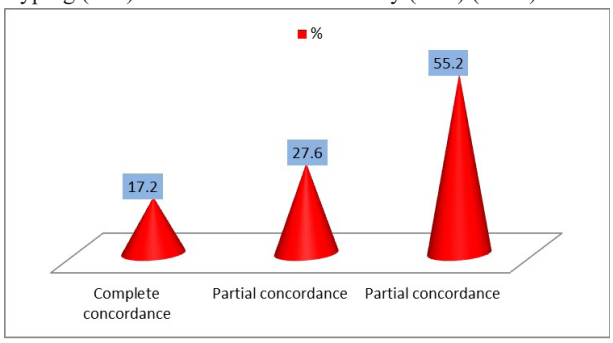

Figure 4: Concordance and discordance between Flowcytometry and Histopathology.

Table 3 shows Concordance and discordance between results of Flowcytometry and Immunohistochemistry (IHC) where out of 17 cases, complete concordance and partial concordance was found in 6 (35.3%) and 6 (35.3%) cases respectively. Discordant result was seen in 5 (29.4%) cases.

Concordance/Discordance	Number	%
Complete Concordance	6	35.3
Partial concordance	6	35.3
Discordance	5	29.4
Total	17	100

Table 3: Concordance and discordance between results of Flowcytometry and Immunohistochemistry (IHC) (n=17).

Immunophenotypic criteria for diagnosis of Classical Hodgkin lymphoma (CHL) in study population are shown in Figure 5 (a). In this study Hodgkin lymphoma was diagnosed by FCI in one case only. This study found an extra population of cells which was CD45 negative. This CD45 negative population was also negative for CD19 and CD3 but was positive for CD30, CD15 and CD40 which was diagnostic criteria for Hodgkin lymphoma by FCI.

Flow cytometric immunophenotyping criteria for B-cell Non-Hodgkin lymphoma (DLBCL)is depicted in Figure 5 (b) where gated cells were negative for CD5 and CD23 but positive for CD45, CD19, CD20, CD22, CD79a, CD79b, FMC7, Bcl-2 with light chain restriction.

Flow cytometric immunophenotyping criteria for T-cell Non-Hodgkin lymphoma (ALCL) has been shown in Figure 5 (c) where the gated cells showed strong reaction to CD45, CD3, CD5, CD30, CD56; medium reaction to CD4, CD8, CD7, BCL-2 but negative to CD10. A CD30 expression along with one or more T-cell markers is characteristics but association of CD56 marker in some cases can confer a poor prognosis in Anaplastic large cell lymphoma (ALCL).

Out of 40 cases 7 (17.5%) cases were non consistent with lymphoma which did not express any markers of lymphoma panel (Figure 5d).

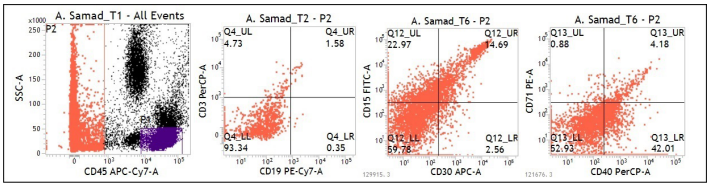

Figure 5a: Flow cytometric immunophenotypic findings in a patient with Classical Hodgkin Lymphoma.

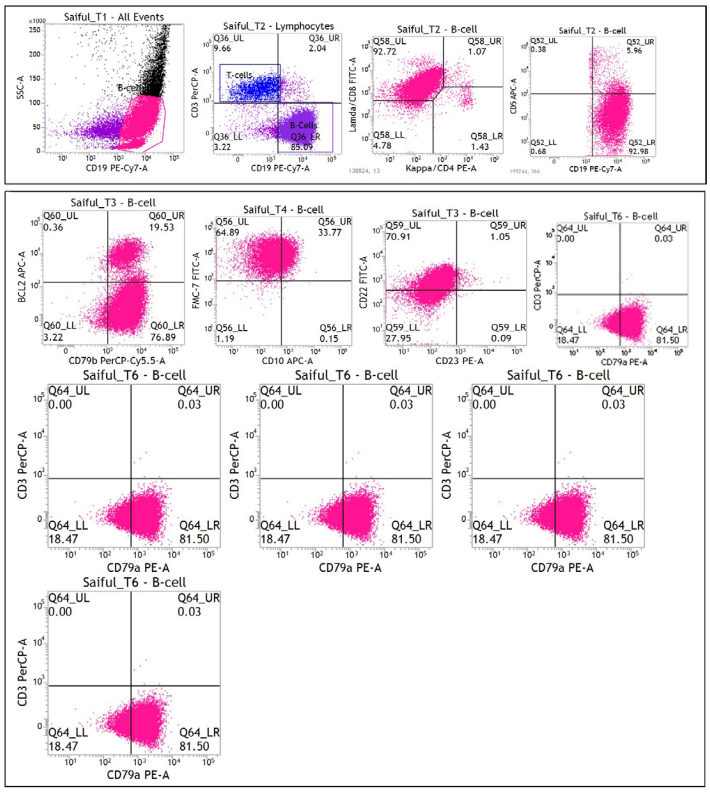

Figure 5b: Flow cytometric immunophenotypic findings in a patient with DLBCL.

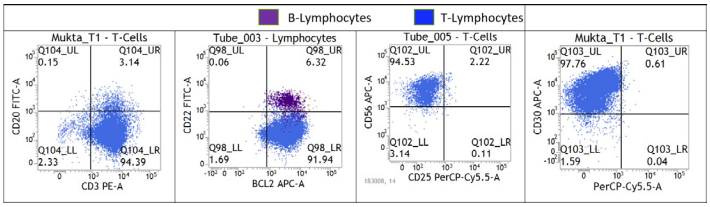

Figure 5c: Flow cytometric immunophenotypic findings in a patient with Anaplastic Large Cell Lymphoma (ALCL).

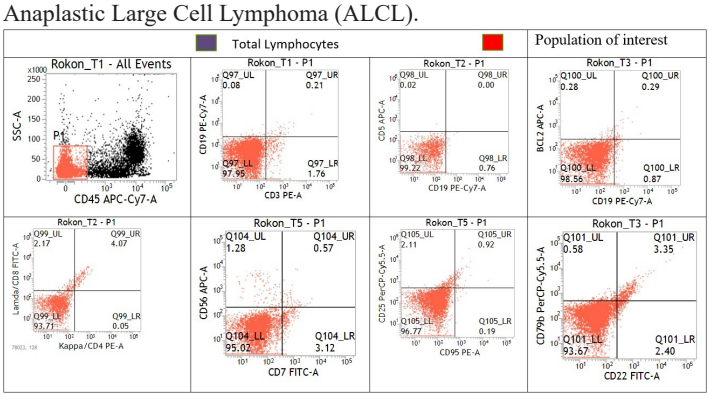

Figure 5d: Flow cytometric immunophenotypic findings in a patient with Not consistent with Lymphoma.

Discussion

Lymphoma can be diagnosed by different methods like fine needle aspiration cytology (FNAC), histopathology, Immunohistochemistry (IHC) and Immunophenotyping by flowcytometry (FCI). In this study, among 40 suspected lymphoma cases, 32 (80%%) cases were diagnosed as lymphoma but 7 (17.5%) cases were not consistent with lymphoma and 1 (2.5%) case was reactive hyperplasia by FCI. Among the 32 cases diagnosed as lymphoma, 31 (96.9%) cases were diagnosed as Non-Hodgkin lymphoma (NHL) and 1 (3.1%) case was diagnosed as Hodgkin lymphoma (HL).

Higher incidence of NHL has been reported from studies at different centers of different ethnic groups. One study reported that 48.8% Non-Hodgkin lymphoma and 4.45% Hodgkin lymphoma with 27.4% reactive process by FCI [11]. Similarly, other study showed 41.3% Non-Hodgkin lymphoma compared to 9.4% Hodgkin lymphoma by FCI. Among the Iranian population, similar picture of 37.9% Non-Hodgkin lymphoma compared to 20.6% Hodgkin lymphoma with 37.9% Reactive hyperplasia has been reported by one study [12].

In this study, the overall concordance between FCI and histopathology (both complete and partial) was 44.8% and discordance 55.2 %. Other study found concordance between FCI and histopathology in 89% of cases which were suspected to be lymphoma. The overall concordance (both complete and partial) between FCI and IHC was 70.6% and discordance was 29.4% in the present study. A study in Texas revealed 97% concordance and 3% discordance between FCI and IHC [13]. In the older studies, concordance and discordance between FCI and other subjective procedures are not so wide. The reason may be of limited CD markers and dye tag monoclonal antibodies to CD markers. With development of large variety of CD markers are now being subjected during the phenotyping. As a consequence, more discordance between FCI and subjective procedures has been emerged. Comparison between FCI and subjective procedures could have been more précise if all the histopathology and immunohistochemistry reports could be available. In this current study, 29 cases who underwent histopathology were available for immunophenotyping while 17 cases from IHC.

Introduction of immunophenotyping by flow cytometry in the diagnosis of lymphoma has brought a fundamental change. Since the identification of different CD markers can easily differentiate B-cell, T-cell and NK cell which is not possible in such précise way by histopathology, because of the subjective nature of the procedure. This is very much evident from our findings that where 24.1% cases were diagnosed as Hodgkin lymphoma by histopathology which was only 3.4% by flow cytometry. A closer result has been reported by Ensani et al. [12] where 20% was Hodgkin lymphoma by histopathology and 27% by FCI; Fromm et al.14 also reported 53% cases by histopathology and 47% cases by FCI.

The situation becomes further complicated in cases of Non-Hodgkin lymphoma subtyping because more than 60 subtypes of NHL has been identified at present. By histopathology, it is almost impossible to detect all subtypes specially T-cell and NK cell type Non-Hodgkin lymphoma, as well as the subtypes vary in disease progress. Flow cytometric immunophenotyping directly recognize the different subtypes associated with disease progression. But in case of histopathology different grades can be reported which can give idea indirectly about the variety of Non-Hodgkin lymphoma.

Besides FCI, Immunohistochemistry is being used for detection of lymphoma by using CD markers. IHC has brought a little advantage over histopathology but due to procedural limitation only few varieties of B-cell and T-cell lymphoma can be identified. It is evident that by IHC limited number of B-cell subtype has been identified while in case of T-cell subtype there has been a big drop in the concordance between IHC and FCI.

A large number of lymphoma cases particularly T-cell subtypes initially represent as reactive process by histopathology, but flowcytometric Immunophenotyping can solve this controversy by detecting specific markers.

Conclusion

FCI from FNA sample can enhance the diagnostic potential and avoid the need for invasive surgical biopsies. Moreover, it can diagnose more Non-Hodgkin lymphoma than Hodgkin lymphoma. Routine immunophenotyping by flow cytometry should be performed in all lymphoma cases which is diagnosed cytomorphologically and histopathologically, for both confirmation and better characterization of disease by typing and subtyping.

Limitations

Small sample size and non-availability of histopathology and immunohistochemistry reports of all the study population were the limitations of this study.

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