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Original Research Article

Immunophenotyping of non-Hodgkin's lymphoma by flowcytometry on fine needle aspiration

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ABSTRACT

Background: Lymphoma represents one of the major health problems all over the world. Flow cytometry (FCM) can be used on fine-needle aspiration cytology (FNAC) from lymph node as an ancillary technique. Aim of the study was to assess the utility of flowcytometry (FCM) in diagnosis and differentiation of reactive hyperplasia and non-Hodgkin's lymphoma (NHL) on FNAC.
Methods: The study was carried out on 50 cases, 25 each of reactive hyperplasia and suspicious or confirmed NHL on FNAC. FCI was performed with a complete panel of antibodies on FACS Canto II FCM.
Results: All 25 cases of reactive hyperplasia on FNAC were polyclonal on FCM. FCM could be performed in 22 cases (88%) out of 25 suspicious NHL and in three cases the material was inadequate on aspirate. Out of 22 cases of NHL on FNAC 17 cases (77.30%) were diagnosed as B-NHL on FCM. Light chain restriction was demonstrated in 15/17 cases. With the help of FCI, 6 cases were diagnosed as small cell lymphocytic lymphoma, one case as mantle cell lymphoma, one case as follicular lymphoma, and 9 cases as B-NHL-NOS. Histopathology diagnosis was available in nine cases and were in concordance to FCM. Sensitivity of combined FNAC and FCM in sub-classification was 77.30% (17/22). Four cases showed discordance between FNAC and FCM.
Conclusion: We concluded that FCM enhances the diagnostic ability of FNAC, playing a crucial role in a rapid and accurate differential diagnosis between reactive hyperplasia, B-NHL and T-NHL.

Keywords: Flowcytometry, Non-Hodgkin's lymphoma, Fine needle aspiration cytology, Reactive hyperplasia

INTRODUCTION

Lymphoma represents one of the major health problems all over the world. It is a common malignancy affecting both children and adults and is continuing to increase rapidly. In lymphoma diagnosis, immunohistochemistry has the advantage that the cells of interest are identified morphologically and it is applicable retrospectively to fixed tissues. However; in this technique there are some limitations like, loss of some cells and/or cellular antigenicity due to fixation, study single marker at a time, permits examination of about 100 cells only and sometimes difficulty in demonstrating immunoglobulin light chains.[1,2]

Now a days flow cytometric immunophenotyping has become a widely used laboratory procedure for diagnosis and sub-typing 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). On the contrary to immunohistochemistry (IHC), flow cytometry (FCM) allows a more precise definition of individual cell types since the cells of interest are identified by a combination of physical characteristics and the use of multiple antibodies directly conjugated with different fluorochromes which allows detection of expression of combination of 2 or 3 antigens on the same cell. It also has the ability to assess monoclonality through detection of

immunoglobulin light chain expression and the results can be available within few hours after receiving the specimen.[1]

The use of fine needle aspiration cytology (FNAC) in the investigation of lymphadenopathy is widely practiced minimally invasive technique. It is safe, relatively painless, simple, rapid and highly cost effective as a first line investigative technique to rule out malignancies and to confirm reactive or infective pathology.[2] FCM can be used on FNAC from lymph node as an ancillary technique for the diagnosis and classification of non-Hodgkin's lymphoma (NHL) through evaluation of lymphoid B- and T-cell antigens and clonality assessment of light chains.[3]

This study was conducted to assess the utility of FCI in diagnosis and differentiation of reactive hyperplasia and NHL on FNAC aspirates and correlating the results of FNAC and FCM with histopathology and immunohistochemistry (IHC) wherever possible.

METHODS

The present prospective study was carried out in Department of Pathology, Pt. B. D. Sharma, UHSR (PGIMS), Rohtak, Haryana over a period of 2 years (2015-2017). A total of 50 cases, 25 each of reactive hyperplasia and suspicious/confirmed cases of NHL on FNAC were included in the study. Complete relevant clinical details age, sex, fever, pain, weight loss, weakness or any other significant history was taken. Physical examination was done for lymphadenopathy and organomegaly. Cases of metastatic non-lymphoid malignant disease and Hodgkin's lymphoma were excluded. Reactive hyperplasia was taken as control for this study.

Specimen preparation

0.5 ml of Roswell Park Memorial Institute (RPMI)-1640 (tissue culture media) was taken in two Eppendorf tubes for each patient. FNAC was done by standard technique using 23-gauge needle and a 10 ml syringe. Smears were prepared using Romanowski stain. A second pass of the needle was done and material collected in RPMI-1640 media for FCI. The specimen was immediately processed for chronic lymphoproliferative disease panel (CLPD) for immunophenotyping (IPT). IPT was performed on 8 color flow cytometer BD FACS Canto II (San Jose, CA, USA) using CD45/SSC, CD19/SSC and CD3/SSC gating. The CD panel used was CD45, CD19, CD3, CD4, CD8, CD56, CD20, CD5, CD10, CD79b, CD23, CD38, CD25, CD11c. CD103, FMC7, CD200, CD2, CD5, CD7, CD57, Kappa, Lambda, TCR-αβ, TCR-γδ and CD30. All analyses and interpretation were carried out using the FACS-Diva software (BD Biosciences).

Results were analyzed for a minimum number of 30,000 events for each cell marker. The expression levels of the individual antigens were described as strong, moderate, and dim, depending on the order of the magnitude by which the fluorescence intensity with their cognate antibodies was higher than the corresponding isotype controls.

Data interpretation

List mode data was acquired on FACS Canto II flow cytometer (BD Biosciences) and analysed by FACS Diva software. More than 20% expression of any gated events on the side scatter/CD 45 plots was considered as positive. Wherever possible, results of FNAC and FCM were correlated with histopathological and IHC results.

RESULTS

There were 33 males and 17 females with male to female ratio 1.9:1. Age range of reactive hyperplasia cases was 60-70 years. Maximum number of cases were in the age group of <30 years (64%) in reactive hyperplasia. All 25 cases of reactive hyperplasia were polyclonal on FCM (Table 1).

Table 1: Distribution of age of total patients (reactive hyperplasia).

Age in years	No. of patients	Percentage
<30	16	64
30-40	4	16
40-50	2	8
>50	3	12
Total	n=25	100

Age range of NHL cases was 20-80 years. Maximum number of cases were in the age group of >50 years (64%) in NHL. FCM could be performed in 22 cases (88%) out of 25 suspicious cases of NHL and in rest of the three cases the material was inadequate because of scanty blood mixed aspirate. Out of 22 cases of NHL on FNAC only 17 (77.30%) cases were diagnosed as B-NHL. Out of 17 cases of NHL, 15 cases showed monoclonality on FCM (Table 2).

Table 2: Distribution of age of total patients (suspicious of lymphoma) on FNAC.

Age in years	No. of cases	Percentage
<30	1	4
30-40	2	8
40-50	6	24
>50	16	64
Total	n=25	100

Nine cases (52.94%) showed lambda restriction, six cases (35.30%) showed kappa restriction, two cases (11.76%) of lymphoma showed no restriction. On subtyping of B-NHL on FCM, small lymphocytic lymphoma (SLL) constituted 6 (35.30%) cases, mantle cell lymphoma (MCL) one (5.9%) case, follicular lymphoma (FL) one (5.9%) case and B NHL-NOS nine (52.94%) cases. Nine cases of NHL

were correlated with histopathology and IHC and results were in concordance with FCM (Table 3).

Out of 22 cases of suspicious of lymphoma 4 cases (18.2%) showed discordance between FNAC and FCM. One case which was suspicious on FNAC was reactive on FCM and histopathology both. Considering histopathology as gold standard, FCM was concordance with histopathology in that case. Three cases which were suspicious of NHL on FNAC were not diagnosed on FCM due to insufficient material for flow (Table 4 and 5).

Table 3: Sub typing of B-NHL on FCM.

FC results	No. of cases	Percentage
SLL	6	35.30
MCL	1	5.90
FL	1	5.90
B NHL-NOS	9	52.94
Total	17	100

SLL=Small lymphocytic lymphoma, MCL=mantle cell lymphoma, FL=follicular cell lymphoma, NHL-NOS=non–Hodgkin lymphoma not otherwise specified

Table 4: No. of cases which showed concordance between FNAC/FCM/histology (total-17/25).

No. of cases	FNAC N=17	FC N=17	Histology (no. of cases which were followed for biopsy; n=9)
1	Suspicious	Mantle	Mantle (1)
1	Suspicious	Follicular	Follicular (1)
6	Suspicious	SLL	SLL (2)
9	Suspicious	B NHL-NOS	B-NHL peripheral type (5)

Table 5: Discordance between FNAC/FCM results and histology results.

No. of cases	FNAC results	Flow results	Histology results
3	Suspicious of chronic lymphoproliferative disorder	Reactive	Could not be followed
1	Suspicious of chronic lymphoproliferative disorder	Reactive	Hodgkin's lymphoma

DISCUSSION

In recent years, FCM has proven useful in the evaluation of mainly lymphoproliferative disorders on samples obtained by surgical specimens or FNAC; therefore, FCM applied to FNAC has been used widely, often replacing classic immunocytochemistry. The possibility of applying a complete panel of antibodies and diagnostic algorithms is the most appreciated advantage of the technique.[4-8] In fact the latest revised European American lymphoma (REAL) classification of lymphoma enhances immunophenotyping and cytologic features of lymphoid populations rather than the conventional nodular or diffuse growth patterns that characterized previous NHL classifications.

The main advantages of FCM immunophenotyping are the timely application of a complete panel of antibodies and contextual triage of fluorescein antibodies with detection of specific antibody expression and co-expression patterns that allow the application of diagnostic algorithm.[9,10] The distinction between reactive and malignant lymphoid proliferations is the most problematic area in lymph node FNAC. This is not surprising, given that excised lymph nodes commonly cause diagnostic difficulty despite the advantage of architectural preservation in biopsy specimens. Aspirate specimens from cases of high-grade lymphoma and Hodgkin's disease may show an obvious cytomorphological abnormality, but the diagnosis of low-grade lymphomas in cytological preparations is most often based on the presence of a relatively monomorphic lymphoid population, contrasting with the typically polymorphous cell pattern seen in reactive proliferations. Therefore, potential cytological misdiagnoses may occur, either in lymphomas that present an apparently admixed cell pattern (false negative cases), or in reactive proliferations in which atypical cells are identified (false positive cases). For these reasons, excision biopsy is advocated by most authors to confirm a primary cytological diagnosis of lymphoma.

The most common age group for reactive hyperplasia was younger population (<30 years) (range 6-70 years) in contrast to NHL where majority constituted >50 years of age (range 24-80 years). Age range in suspicious of lymphoma cases was 20-80 years. These findings were similar to study conducted by Dey et al on 48 cases.[11]

Studies conducted by Dey et al and Barroco et al showed male predisposition which was similar to our findings showing male: female ratio to be 1.9:1.[11,12]

Barroca et al and Dey et al have found that most common site for lymphomas was cervical region, this was in concordance with our finding (i.e. 68%) followed by axillary and inguinal lymphadenopathy (Table 6).[11,12]

Zeppa et al in a study of 307 cases of lymph nodal and lymph nodal lymphoproliferative disorders and Dey et al in his work found most common lesion on FNAC with

FCM confirmation as B-NHL NOS.[11,13] Similar findings were seen in our study showing B-NHL NOS followed by SLL, MCL and FL. These results were confirmed histopathologically. FCI was also helpful in sub-classification of most of the cases of NHL. It was particularly helpful in sub-classification of low grade B-NHL. The FNAC of small lymphocytic lymphomas, mantle cell lymphomas and low grade follicular lymphomas are difficult to differentiate by cytomorphology alone.

Barroca et al in 2005 found that there was discordance between FNAC and FCM in 4 cases out of 113 (i.e. 4.4%). We also found FNAC/FCM discordance in 4 cases out of 25 cases (i.e. 16%). But these cases could not be further followed by histopathological examination. Our results were almost 2 times higher than that of Barroca et al study.[12] It can be attributed to small sample size and misdiagnosis on FNAC. Our results were in concordance with those of Medas et al who reported 4 cases of reactive hyperplasia on FCM those were suggestive of lymphoma on FNAC.

In the present study we applied FCI to diagnose and sub-classify NHL on FNAC smears according to WHO classification. Expression and co expression of different CD markers were helpful in their study for subtyping NHL. There are a few available studies in this aspect. Zeppa et al in a study were able to sub-classify 70 cases of NHL among the 115 cases of NHL with small and medium sized cells.[14] Study conducted by Dey et al on 48 cases out of which 38 (79%) cases were sub classified as per World Health Organization (WHO) classification with 100% specificity and 83.8% sensitivity.[11]

In a similar study, Siebert et al were able to subtype 29 out of 38 (76.3%) cases of NHL with the help of FCI.[15] They also highlighted that adjunctive FCI and FNAC is potentially practicable in a community hospitals and can help direct lymphoma therapy. Mourad et al were also able to sub-classify large number of cases according to WHO sub-classification with the help of FCI.[16] Mayall et al in a recent paper showed that FNAC along with FCI were very helpful to diagnose and sub-classify B-NHL but FCI had little use for T cell NHL in their study.[17] Our study was also comparable with the above mentioned studies as we also were able to sub-classify 68% of histopathology proven cases and large number of small cell B-NHL. It was not very informative in large cell type of B-NHL. In these cases, cytomorphologic feature are helpful in sub-classification of NHL. FCI is also likely to miss difficult cases such as ALCL. Monoclonality was difficult to prove in T-NHL on FCI; however aberrant expression of T cell markers and predominant expression of CD4 or CD8 indicated T cell NHL.

CONCLUSION

We conclude that, the FCI contributes significantly to and are consistent with the final tissue diagnosis in the majority of studied cases (sensitivity 80.95%, specificity 100%). The false negative results of FCI could be attributed to the presence of heterogeneous populations of lymphocytes that might be present in special situations such as partial involvement of the lymphoid tissue by lymphoma cells, the presence of a follicular lymphoma with normal lymphoid cells in between the neoplastic follicles, or the presence of numerous residual non-neoplastic lymphocytes among the neoplastic cells of diffuse lymphomas as in T-cell-rich B-cell lymphoma. Thus, in highly suspicious cases IHC is still required if no FCI abnormalities were detected. However, FCI has a definite role in detection of monoclonality (light chain) of NHL.

In conclusion FNAC along with FCM is an easy and accurate alternative to surgical biopsy for lymphoproliferative disorder. This technique is highly accurate in diagnosing and sub classifying NHL and relatively less invasive and cheaper. In the near future, it may become the mainstay for the diagnosis and follow-up of NHL.

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