original article

Fine needle aspiration cytology in the evaluation of lymphoid lesions: a retrospective study of the utility of flow cytometry in conjunction with morphology

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BACKGROUND AND OBJECTIVE: Fine needle aspiration (FNA) cytology, in conjunction with flow cytometry, is now widely used as a reliable and accurate method for the assessment of various lymphoid lesions, especially for lesions situated in odd locations where obtaining biopsy and monitoring for recurrence in previously diagnosed cases of lymphoma are difficult. The objective of this study was to determine the utility of FNA and immunophenotyping in the assessment of lymphoid lesions, and to find whether flow cytometry is more useful in the evaluation and subclassification of the small cell morphology group of lymphomas than in the large cell morphology group of lymphomas.

DESIGN AND SETTING: Retrospective analysis of patients diagnosed with lymphoma over at a 5-year period.

PATIENTS AND METHODS: All 175 FNA cases were followed carefully either clinically or histologically for at least 5 years. We compared the utility of flow cytometry in the diagnosis of small cell morphology lymphomas to large cell morphology lymphomas.

RESULTS: Flow cytometry was performed on 72 of 175 (41%) of FNA specimens clinically suspicious of lymphoma. The excisional follow-up biopsy was obtained in 78 of 175 (44.5%) cases. Based on cytomorphologic evaluation, 82 cases (47%) were considered negative, 34 cases (19%) were considered atypical, 32 cases (18%) were positive for NHL-small cell morphology, 21 cases (12%) were positive for non-Hodgkin lymphoma (NHL)-large cell morphology, 3 cases (2%) were positive for NHL, and 3 cases (2%) were nondiagnostic. Immunophenotyping utilizing flow cytometry was the diagnostic parameter in 28 of 32 cases (88%) of the NHL-small cell morphology group and in 11 of 24 cases (46%) of the NHL-large cell morphology/Hodgkin lymphoma group.

CONCLUSIONS: Immunophenotyping by flow cytometry is more essential for the accurate evaluation and classification of small cell morphology than large cell morphology lymphoid lesions in FNA cytology.

Fine needle aspiration (FNA) cytology is a widely used diagnostic method for the assessment of various benign and malignant lymphoid lesions. It has been proven to be an accurate, reliable, and cost-effective method of diagnosing and subclassifying malignant lymphoma. It is routinely performed as the primary diagnostic workup for patients with lymphadenopathy, documenting recurrent disease, for staging, and for obtaining material for ancillary studies.[1-4] The classification of non-Hodgkin lymphoma (NHL) emphasizes the diagnostic importance of cytomorphology, immunophenotyping, and molecular findings in addition to histology.[5,6] Previous studies have addressed the usefulness and accuracy of immunophenotyping using flow cytometry (FC) in the FNA diagnosis of lymphoid lesions.[4,6,7] There has not been an emphasis on the usefulness of FC when comparing small cell morphology and large cell morphology lymphoid neoplasia

PATIENTS AND METHODS

This was a retrospective analysis of 175 FNAs in 167 patients with concurrent FC results, conducted at Scott

and White Memorial Hospital, Texas A&M College of Medicine, Temple, TX, USA, for a 5-year period. Correlation was made either histologically in cases where a follow-up tissue biopsy was available or clinically by closely monitoring the patient's clinical records for at least 5 years in cases where no follow-up tissue biopsy was obtained. FNA cases with no histologic or clinical follow-up were excluded from our study.

All cases were diagnosed using the new World Health Organization (WHO) classification of malignant lymphoma. The small cell morphology group (**Figure 1**) included small cell lymphocytic lymphoma; follicular lymphoma, grade 1; follicular lymphoma, grade 2; marginal zone lymphoma, mantle cell lymphoma, and small cell (not otherwise specified) lymphoma. The large cell morphology group (**Figure 2**) included follicular lymphoma (grade 3), large cell lymphoma, and Hodgkin lymphoma (HL) (**Figure 3**).

FC (**Figure 4**) was performed in 72 of 175 cases (41%). FNA specimens received for FC wore filtered and washed as required. A cytospin was prepared for each specimen, which was reviewed by a pathologist, prior to selecting the panel of antibodies for analyzing the cells from the FNA specimen. The above panel was tailored to the special requirements such as patient history and/or number of viable cells available in the specimen. The instrument used was the Becton Dickinson FACScan (BD Biosciences, San Jose, CA, USA), 3-color staining/analysis. Approximately 0.5 million cells are allocated to each tube to which the appropriate antibodies are added. Prior to staining the kappa/lambda tubes with antibodies, these cells are washed 2 to 3 times with phosphate buffered saline (PBS) to remove any free-floating kappa or lambda that may be present. The tubes are incubated for 15 to 30 minutes at room temperature and protected from light. After initial incubation, red blood cells are lysed in Becton Deckinson's FACS Lysin Solution (BD Biosciences). After a 5- to 10-minute room temperature incubation to lyse red cells, each tube is centrifuged to pellet the cells. The lyse solution is decanted and the cells are washed with PBS. The PBS is decanted and the cells were fixed in 0.5 mL of a 0.5% formaldehyde fixative solution. The panel of tubes was then collected on the FACScan. Ten thousand events were collected from each tube after a threshold is established to exclude debris. The data collected were then analyzed using Becton Dickson Cell-Quest software (BD Biosciences). The CD45 data were used to establish all populations in the specimen by gating from a CD45 fluorescein isothiocyanate conjugated versus side scatter (SCC) dot plot. The data from the remaining tubes (excluding propidium iodine) were analyzed
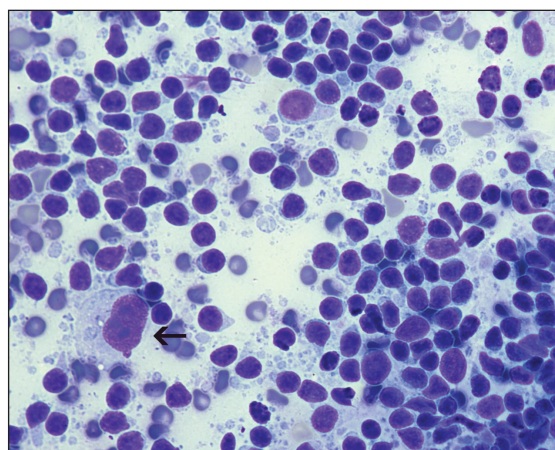

Figure 1. Non-Hodgkin lymphoma, small cell morphology (Pap stain 20×).
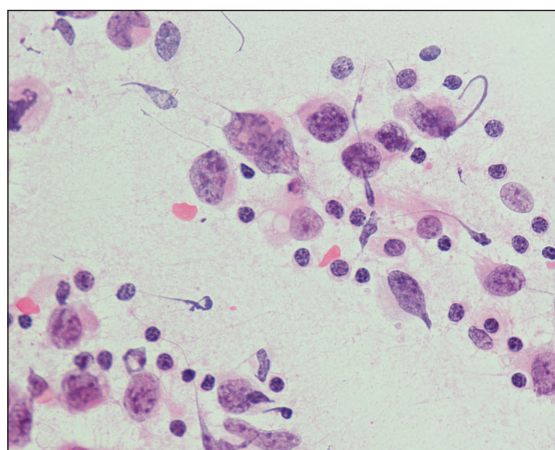

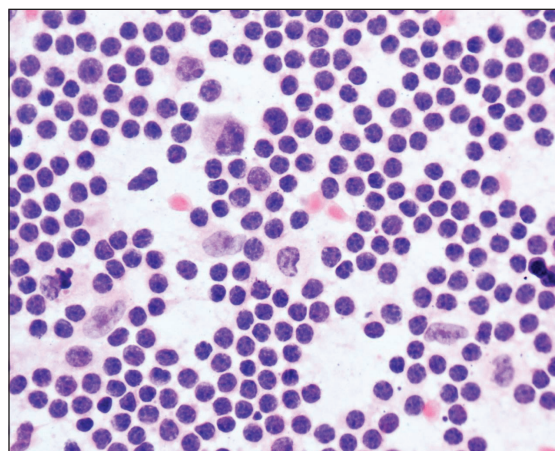**Figure 2.** Non-Hodgkin lymphoma, large cell morphology. Hematoxylin and eosin stain (40×).

Figure 3. Non-Hodgkin lymphoma, large cell morphology. Hematoxylin and eosin stain (40×).

by gating CD45 prep versus SCC dot plot. Analyzing at least 10 000 ungated events derives the viability.

RESULTS

The aspiration material was obtained either from superficial locations in 139 of 175 cases (79%) or from deep locations, with the guidance of image studies, in 36 of 175 cases (21%). An excisional follow-up biopsy was obtained in 78 of 175 cases (44.5%). Review of clinical follow-up treatment of all positive cases with FNA results with or without the follow-up biopsy was performed. FNA diagnoses were as shown in **Table 1**. The age of the patients ranged from 6 to 98 years (mean age 60 years). The female:male ratio was 1.4:1 (male: 71 cases, female: 104 cases). The primary sites were nodal (n=148) and extranodal (n=27). Among the 175 FNAs, a history of a previous diagnosis of a malignant lymphoma was available in 40 cases. A total of 16 of 32 in the small cell group had a previous diagnosis of lymphoma and 10 of 24 in the large cell group had a previous diagnosis of lymphoma. Over half of the cases (29 out of 56) did not have a previous diagnosis of lymphoma. FC was performed in 72 of 175 cases (42%). It was diagnostic in 28 of 32 cases (88%) of the small cell group and in 11 of 24 cases (45 %) of the large cell/Hodgkin group.

The follow-up tissue biopsy in 78 of 175 (44.5%) of the cases is shown in **Table 2**. Indications for the follow-up biopsy were: atypical FNA diagnosis, first time/primary diagnosis of lymphoma, HL, high clinical suspicion for lymphoma in benign aspirates, and finally the lack of confidence by some oncologists in accepting an FNA diagnosis of a hematopoietic malignant neoplasm. For the small cell group (n=32), a follow-up biopsy was performed in 13 cases. For the large cell group (n=21), a follow-up biopsy was obtained in 5 cases. Four cases reported by FNA as negative were determined by the follow-up biopsy to be false-negative FNA results. Three of the false negatives were from the large cell group and the fourth was from the HL group. Most of the atypical diagnostic category cases (30 of 34) were followed by open biopsies, which revealed a positive diagnosis of malignant lymphoma in 23 of 30 cases (75%). All 3 cases with nondiagnostic FNA results were followed by open biopsies, 2 resulting in a final diagnosis of small cell group lymphoma and 1 resulting in a final diagnosis of negative for neoplasia.

The small cell group included 32 cases (**Table 3**). A previous diagnosis of lymphoma was reported in 16 cases. In 19 cases, no follow-up biopsy was reported. In 6 cases, neither a previous diagnosis of lymphoma nor follow-up biopsies had been reported, and the treatment depended on FNA results. The 13 follow-up biopsies in
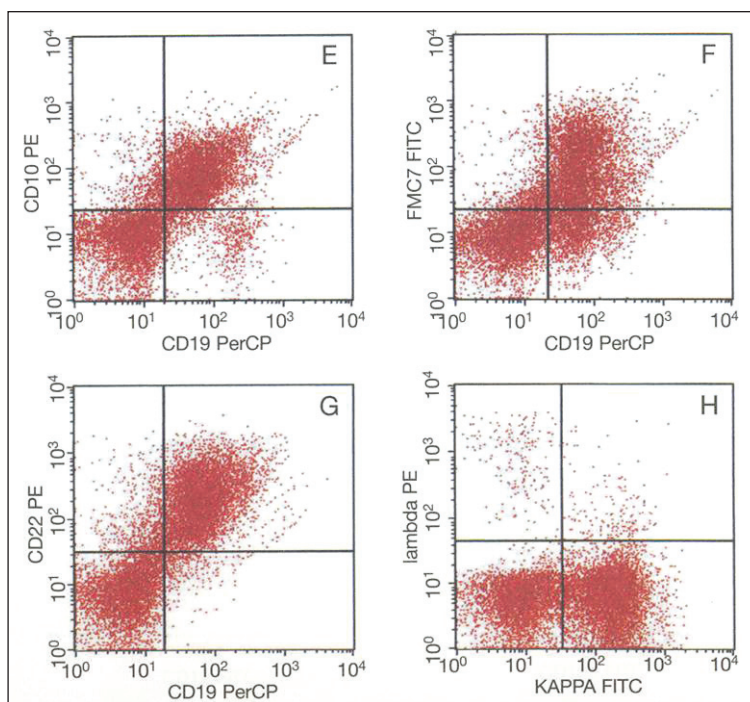

Figure 4. Flow cytometry, follicular lymphoma, showing positivity for many B-cell markers (CD19, CD22, FMC7), follicle center cell marker (CD10), and evidence of monoclonality (kappa light chain restriction).

Table 1. Fine needle aspiration (FNA) diagnosis by flow cytometry and/or cytomorphology.

FNA diagnosis categories	Number of cases
Nondiagnostic	3 (2%)
Negative	82 (47%)
Atypical	34 (19%)
Positive small cell	32 (18%)
Positive large cell	21 (12%)
Hodgkin disease	3 (2%)
Total	**175 (100%)**

this group confirmed by histopathology subclassification of NHL in 12 of 13 cases were as follows: small lymphocytic lymphoma (n=2), follicular lymphoma, grade 1 (n=6), follicular lymphoma, grade 2 (n=1), mantle cell lymphoma (n=3), and small cell, not otherwise stated (n=1). A discrepancy was observed in the subclassification of one case in which the FNA result was small cell group follicular lymphoma, grade 2, while the biopsy result was follicular lymphoma, grade 3.

The large cell group included 21 cases. There was a follow-up biopsy in 5 of 21 cases; all represented a first-

Table 2. Follow-up biopsy results of previous fine needle aspiration (FNA) diagnoses.

FNA diagnosis	Follow-up biopsy diagnosis					
	Not done	Negative	Atypical	Small cell morphology lymphoma positive low grade	Large cell morphology lymphoma positive high grade	Hodgkin lymphoma
Nondiagnostic	0 (0%)	1 (33%)	0 (0%)	2 (67%)	0 (0%)	0 (0%)
Negative	57 (70%)	18 (22%)	3 (4%)	0 (0%)	3 (4%)	1 (1%)
Atypical	4 (12%)	7 (21%)	0 (0%)	9 (26%)	8 (24%)	6 (18%)
Positive small cell morphology lymphoma	19 (58%)	0 (0%)	0 (0%)	13 (39%)ᵃ	0 (0%)	0 (0%)
Positive large cell morphology lymphoma	16 (76%)	0 (0%)	0 (0%)	0 (0%)	5 (24%)	0 (0%)
Hodgkin disease	1 (33%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (67%)

ᵃOne case of follicular lymphoma (grade 2) by FNA was upgraded to follicular lymphoma (grade 3) on the larger biopsy due to sampling differences.

Table 3. Fine needle aspiration (FNA) diagnosis by flow cytometry (FC) and cytomorphology.

FNA diagnostic categories	FC positive	FC negative	FC not performed
Nondiagnostic	0 (0%)	1 (33%)	2 (67%)
Negative	0 (0%)	10 (12%)	72 (88%)
Atypical	0 (0%)	18 (53%)	16 (47%)
Positive small cell morphology lymphoma	28 (88%)	1 (3%)	3 (9%)
Positive large cell morphology lymphoma	11 (52%)	3 (14%)	7 (33%)
Hodgkin lymphoma	0 (0%)	0 (0%)	3 (100%)

time NHL diagnosis, and the oncologists preferred obtaining a confirmative tissue biopsy diagnosis. In all of the cases, the biopsy result confirmed the FNA diagnosis. In 16 of 21 cases, no follow-up biopsy was reported, but a previous diagnosis of lymphoma was reported in 8 of 16 cases. In 8 cases, which had neither a previous diagnosis nor follow-up biopsies, the treatment depended solely on FNA results. There were 3 cases of HL diagnosed by FNA cases. One of these cases had a previous diagnosis of HL. FNA results of HL in the remaining 2 cases were confirmed by a follow-up biopsy.

The atypical group included 34 cases. A follow-up excisional/incisional biopsy was reported in 30 of 34 cases. Pathologists and clinicians prefer obtaining a tissue biopsy in each case of atypical FNA diagnosis as there is a high probability of lymphoma diagnosis in the follow-up biopsies. The follow-up biopsies revealed the following: benign (n=7); small cell group (n=9), fol-

licular lymphoma, grade 1 (n=3), follicular lymphoma, grade 2 (n=3), marginal zone lymphoma (n=1), small cell group, not otherwise stated (n=2); large cell group (n=8); and HL (n=6).

Sensitivity for NHL (with positive or atypical results considered as true positives) was 81% and sensitivity for NHL and HL combined was 83%. The false-negative rate was 7.6%. Specificity for NHL was 100% (calculated from positive or negative biopsies, with atypical or nondiagnostic biopsies not included). The false-positive rate in our study was 0%. Accuracy for NHL in our study was 87% (confidence interval 76-95%) and accuracy for NHL and HL combined was 87% (confidence interval 77-94%).

DISCUSSION

The initial diagnosis of lymphoma by FNA is challenging and, in the past, was considered controversial.[8]

The combination of FNA and immunophenotyping by FC has been increasingly accepted as a method for primary diagnosis of NHL. The new Revised European-American Lymphoma Classification/WHO classification places greater emphasis on individual cell morphology combined with immunophenotypic classification, whereas earlier classification schemes emphasized histologic architecture.[9] A review of the published studies reveals the usefulness of this approach in studies that have correlated histology and clinical follow-up with initial FNA diagnoses, supplemented with FC. In one study, 41 FNA samples were obtained and classified as lymphoma, with 25 cases confirmed as lymphoma on a follow-up biopsy and 22 of 25 confirmed to match the subclassification. The discrepancies were because of grading in follicular lymphoma, Hodgkin disease, and T-cell lymphoma.[5] Another prospective study of image-guided percutaneous biopsies for the primary diagnosis of lymphoma showed that FC was used in 10 of 43 cases (23%) and immunohistochemistry was used on core biopsies in 33 of 43 cases (77%). A diagnostic result was obtained in 37 of 43 cases (86%), and 36 of 43 cases (84%) were treated according to the diagnosis rendered. It is clear that FC plays a crucial role in the FNA diagnosis and subclassification of lymphoma, especially the small cell group.[4,6,10] The utility of FC according to the cell size was not emphasized in these previous reports.

Immunophenotyping of lymphomas utilizing FC has long been recommended.[8] Immunophenotyping of lymphomas can be performed in a variety of ways, including FC and immunohistochemistry of cytospin preparation from the FNA material, immunohistochemistry of a cellblock preparation from the FNA material, or immunohistochemistry of core needle biopsies. Immunophenotyping of FNA specimens by FC and immunohistochemistry on cytospin preparations have been compared and, in the past, were felt to be equally reliable. The disadvantage of FC was felt to be the lack of direct correlation of cell morphology to the immunophenotyping results. FC was felt to have the advantages of rapid results, automation, objective statistics and precision, and multiple parameter evaluation. Cytospin preparations were considered to allow better evaluation of cell morphology with the correlation with the immunophenotype.

FC is very useful in the diagnosis of the small cell group with well-defined phenotypes used in their classification scheme.[9] It has been reported that FC was useful and, in many instances, necessary for the diagnosis of indolent lymphomas (which are primarily an NHL with small cell morphology appearance) in 23

out of 23 cases. Two indolent small cell morphology lymphomas, which were not subtyped correctly, were diagnosed as mantle cell lymphomas, when followed up with the lymph node biopsy.[11] The diagnosis of mantle cell lymphoma based on morphology alone is known to be difficult, but recent studies have reported that mantle cell lymphoma can be separated from chronic lymphocytic leukemia (CLL) by FC, since CD23 negative expression is seen in only mantle cell lymphoma. A small proportion (5%) of mantle cell lymphomas were reported to have an overlap with CLL and displayed a weak expression of CD23.[10]

The diagnosis of large cell lymphoma by FC can be difficult because of frequent apoptosis, cell fragility, necrosis, high mitotic rates, and difficulty in gating on appropriate cell populations. There is also the possibility that the lymphoma may not show a diagnostic immunoglobulin light chain restriction phenotypic expression by FC. In a review of 51 cases of large cell lymphoma 4 cases (8%) failed to show monoclonality.[12] Another study has shown that FC was useful or necessary in 11 of 15 large cell morphology lymphomas and HL in FNA. FC was not considered necessary in HL, was considered misleading in 2 large B-cell lymphomas, and only considered necessary in 3 cases of large B-cell lymphoma. They reported no false positives and 3 false negatives. The therapy for both indolent and large cell morphology lymphomas was instituted in 32 out of 36 of the patients on whom follow-up was available, based on FNA and FC only.[11]

Immunohistochemical stains on cytospin material, cellblock material, or core needle biopsy may be useful in special cases of large cell morphology hemato/lymphoid lesions, such as anaplastic large cell lymphoma with an immunophenotype of CD30+, CD15–, S100– and cytokeratin–.[13] The drawbacks of FC only on FNA samples for lymphoma have been listed previously as loss of architectural features (such as proliferative centers in small lymphocytic lymphoma), a few diagnostic cells with HL, lack of sufficient cells for FC without on-site evaluation of specimen as it is being acquired for determining sufficient cell viability, and sampling problems inherent to any small biopsy technique.[11]

Our study demonstrates that FNA continues to be a highly sensitive technique for the diagnosis of lymphoma. It is highly definitive and specific for subclassification of lymphoma when FC is performed and considered diagnostic. All atypical diagnoses by FNA should have a careful follow-up, as 68% of the atypical group in this study were eventually diagnosed as lymphoma. The review of clinical follow-up confirmed that all 20 cases of positive FNA results for neoplasia with

a follow-up biopsy, which were positive for neoplasia, would have received the same treatment for their hematolymphoid neoplasia if only the FNA diagnosis had been used. In 14 of 26 positive cases of NHL, the FNA was the only material available to make the primary diagnosis and provide treatment for the patient. This corresponds with previous reports of FC becoming accepted as the definitive diagnosis of lymphoma.[14] FC is essential for the accurate evaluation of small cell morphology lymphoma, with FC being the diagnostic parameter in 28 of 32 cases (88%). FC is useful for large cell morphology/HL, being the diagnostic parameter in 11 of 24 cases (46%), but additional studies (immunohistochemistry on cellblock, cytospins, or core needle and molecular biology) may be required to increase the diagnostic accuracy of this group. This may require an on-site evaluation of the material at the time of procurement to triage the material efficiently.

Whenever possible, preparation of cytospins from FNA material for immunostaining would be helpful in supporting the cytomorphologic diagnosis in cases when FC is not definitively diagnostic. Additionally, FNA results of lymphoid lesions, as in all body sites, should be interpreted in correlation with the clinical findings. This is demonstrated by the performance of follow-up excisional biopsies in 4 cases reported as negative on FNA. Sampling error and/or partially involved lymph nodes may be a contributing factor to false-negative results.

In conclusion, our study re-emphasizes the important role that can be played by FNA in the diagnosis of lymphoma. It can be highly definitive and specific for subclassification of lymphoma when FC is concurrently performed. FC has a crucial importance in the FNA diagnosis and specific subtyping of lymphoma, especially the small cell group type.

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