

# Flow Cytometric Analysis of Deep-Seated Lymph Nodes

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## ABSTRACT

*Flow cytometry (FC) immunophenotyping is an important tool in the evaluation of lymphadenopathy and is widely used in the diagnosis of non-Hodgkin's lymphomas (NHLs) on fine-needle aspirates of lymph nodes and extranodal sites. Because at least 80% of NHLs are of B-cell type, detection of immunoglobulin (Ig) light-chain-restriction is the most commonly used method for confirmation of monoclonality. The aim of our study was to evaluate usefulness of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) for FC analysis from deep-seated lymph nodes and to compare results of FC clonality analysis to cytomorphologic diagnosis of sampled lymph nodes. For cytological diagnosis direct smears were made, selected slide was stained for rapid-on site evaluation procedure. Sixteen patients with suspected NHL of deep-seated lymph nodes obtained by EUS-FNA were submitted for FC clonality analysis using four-color multi-parameter flow cytometry stained with kappa /lambda/CD19/CD45. Clonality analysis was performed on 11 samples. Monoclonality was demonstrated in seven of 11 cases cytologically diagnosed as NHL and four of 11 cases cytologically diagnosed as benign were polyclonal. Our results show that EUS-FNAC with FC is a sensitive and specific tool in the diagnosis of deep-seated B-NHL. Cytologic diagnosis combined with FC clonality analysis can be performed in majority of cases and may eliminate need for open biopsy in some cases.*

**Key words:** flow cytometry, fine-needle aspiration cytology, endoscopic ultrasound, lymph node

## Introduction

Flow cytometry (FC) immunophenotyping is an important tool in the evaluation of lymphadenopathy and is widely used in the diagnosis of lymphoproliferative disorders including non-Hodgkin's lymphomas (NHLs) on fine-needle aspirates of lymph nodes and extranodal sites. Because at least 80% of NHLs are of B-cell type, detection of immunoglobulin (Ig) light-chain-restriction is the most commonly used method for confirmation of monoclonality<sup>1-4</sup>. This ability to distinguish between B-cell lymphoma and reactive lymphadenopathy makes FC clonality analysis a valuable addition to FNA cytomorphology. FNAC is easily performed on palpable lymph nodes<sup>5,6</sup>. However, the evaluation of deep-seated, nonpalpable lymphadenopathy was technically challenging and computerized tomography (CT) guided-FNA or ultrasound guided FNA

were used when possible<sup>7</sup>. The introduction of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) in the past decade enabled us to reach mediastinal and deeply located abdominal lymph nodes and sample enough material needed for morphologic diagnosis, as well as for ancillary techniques such as FC clonality analysis, immunocytochemistry and molecular and genotypic analysis<sup>8,9</sup>. Fresh cell aspirates with enough cells are required for adequate FC clonality evaluation. On site cytopathologist can judge adequacy of material and make preliminary diagnosis requesting additional sampling for ancillary methods<sup>8</sup>. The aim of our study was to evaluate usefulness of EUS-FNA for FC analysis from deep-seated lymph nodes and to compare these results to cytomorphologic diagnosis of sampled lymph nodes.

**Materials and Methods**

FC analysis was performed on 16 EUS-FNA lymph node samples obtained from January 2007 to May 2009. EUS-FNA specimens of Hodgkin’s lymphoma or metastases were not sampled for FC. All EUS-FNA procedures were performed in the presence of a cytopathologist in the endoscopy suite<sup>10</sup>. Indications for additional sampling for FC were either based on endosonographical appearance suggestive for NHL, or there was clinical suspicion for NHL which could not be excluded on site. Endosonographically identified lesions were sampled with the 22-gauge FNA needle inserted through the working channel of the echoendoscope. When the tip of the needle was seen inside the lesion, the stylet was removed and constant negative pressure was applied while doing back and forth movements. Direct smears were made and selected slide was stained with Hemacolor (Merck) for ROSE procedure (rapid-on site evaluation). ROSE includes rapid staining of chosen cytological slide and on-site microscopic examination of aspirated material. When preliminary cytomorphologic diagnosis was probable lymphoma, additional needle passes (2–4) were done and aspirated material was placed in saline for FC analysis. Cytological slides were subsequently stained according to May-Grünwald-Giemsa for final cytological diagnosis. Clonality analysis by four-color multiparameter flow cytometry was performed on a Beckman Coulter Cytomics FC 500 using CXP software. All samples were stained with monoclonal antibodies kappaFITC/lambdaPE/CD19CyQ/CD45PC7 immediately after the EUS-

**TABLE 1**  
SUMMARY OF PATIENTS CHARACTERISTICS

Patients	16
Gender	
Male	8
Female	8
Age	
Minimum	30
Maximum	82
Median	56
Sites of deep-seated lymph nodes	
Abdominal + peripancreatic	12
Mediastinal	4

-FNA procedure and analyzed within one hour. Clones of monoclonal antibodies used were: kappa FITC (clone Poly Ig), lambda PE (clone Poly Ig) and CD19 CyQ (clone HD-37) and CD45 PC7 (clone J.33). Depending on the cellularity of the sample, monoclonality expression and morphological diagnosis, additional number of antibody combinations, such as CD4, CD8, CD5, CD20, CD23, CD10 can be used for subclassification, which is not included in this study<sup>11</sup>. Analysis of samples was performed using a combination of gating strategies. Lymphoid cells were identified using a combination of CD45 *vs.* side-scatter gating to exclude debris, dead cells and non-lymphoid population. Five thousand to 10.000 events were

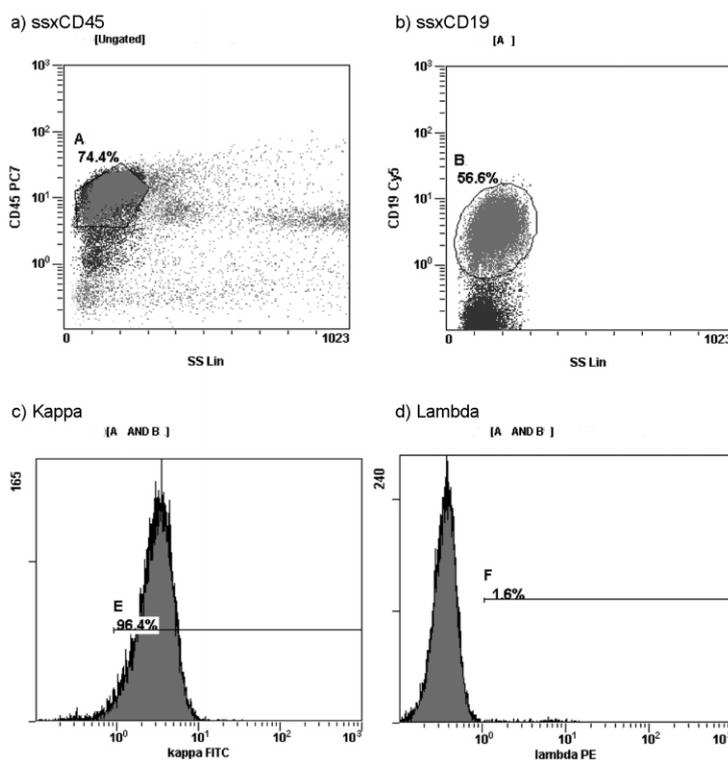


Fig. 1. Gating strategy: a) *ssxCD45*, b) *ssxCD19*, c) *kappa FITC*, d) *lambda PE*.

**TABLE 2**  
COMPARISON OF FLOW CYTOMETRIC CLONALITY ANALYSIS AND CYTOMORPHOLOGIC DIAGNOSIS

FC analysis	No of cases	Cytomorphological diagnosis	No of cases
Inadequate	4	Benign lymph nodes	3
		Suspected NHL	1
Adequate but CD45-	1	Malignant tumor (neuroendocrine)	1
Monoclonality	7	NHL	7
Polyclonality	4	Benign lymph nodes	4
Total	16		16

NHL – non-Hodgkin's lymphomas

required for each tube and samples with at least 30% of CD45 positive cells were considered adequate for further analysis using CD19 vs. side scatter gating to evaluate B-cell lineage, followed by kappa or lambda clonality. In addition to morphology and FC analysis, immunocytochemistry was performed when necessary. Gold standard for diagnosis was biopsy, when available, and clinical follow-up (especially for benign cases).

## Results

After rapid on site evaluation of cytologic smears, samples from 16 patients suspicious for NHL were submitted for FC clonality analysis. There were eight male (50%) and eight female (50%) patients; aged from 30 to 82 years (median 56 years). Six patients had undergone procedure for suspected pancreatic lesions, 6 for suspected abdominal and 4 for mediastinal masses. A summary of patients characteristics is presented in Table 1.

In five out of 16 cases FC clonality assay was not performed. Four out of 5 were inadequate for diagnosis due to scant cellularity of aspirated material. It is worth mentioning that three of them were cytologically diagnosed as benign, and only one was cytologically diagnosed as suspicious for low-grade NHL. One out of five samples had enough cells for clonality assay but these large granular cells were CD45 negative, so the assay was not performed. In this case immunocytochemistry was performed on cytospin-liquid sample. Finally, this case was cytologically diagnosed as malignant tumour with neuroendocrine differentiation. On subsequent core needle biopsy specimens and confirmed by cytogenetic analysis on bone marrow aspirates final diagnosis of PNET was established. Monoclonality was demonstrated in 7 of 11 adequate samples with 6 cases demonstrating monoclonal kappa light chains and one case demonstrating monoclonal lambda chains (Figure 1). Five of seven cases had a monoclonal homogenous population of B-lymphocytes, and in two out of seven cases we found homogenous population of T lymphocytes with small population of large B monoclonal lymphocytes. All monoclonal cases were cytologically diagnosed as NHL. Polyclonality was demonstrated in four of 11 cases. All polyclonal cases were cytologically diagnosed as benign reactive lymphadenopathy or granulomatous reaction.

A summary of these findings is presented in Table 2.

## Discussion and Conclusion

FNAC is recognised as fast, inexpensive and reliable method for evaluation of lymphadenopathy and EUS-guided FNA offers advantages of accessing sites that are difficult to reach by other methods (such as the retroperitoneum, abdominal organs and mediastinum)<sup>12,13</sup>. Diagnosis of lymphoma, regardless of its primary site, offers a several challenges to the cytologist. Low grade lymphoma can be difficult to recognize, follicular hyperplasia may be morphologically indistinguishable from follicular lymphoma (FCL) and high grade lymphoma may look like a number of other malignant tumors, especially small blue round cell tumours<sup>14,15</sup>. Using FC clonality analysis to demonstrate lymphoid lineage and monoclonality of B cell NHLs help us to establish correct diagnosis. In our small series of EUS-FNA sampled lymph nodes, all monoclonal cases were cytologically diagnosed as lymphomas on routinely MGG stained slides. We also showed that all polyclonal samples were cytologically diagnosed as benign reactive lymphadenopathies, which was confirmed by clinical follow up. Using FC analysis of samples obtained by this method had some limitations including adequacy of aspirates regarding its cellularity. Four out of 16 samples were inadequate due to insufficient number of cells for FC analysis. Although presence of cytologist during EUS procedure ensures adequacy of aspirated material for morphologic diagnosis, these samples were not cellular enough for FC analysis despite additional passes made at the time. We also noted larger amount of cellular debris and presence of CD45 negative cells (contamination with epithelial and other cells from gastrointestinal tract) so we used combination of CD45 vs. side-scatter gating to identified lymphoid cells and exclude debris, dead cells and non-lymphoid population. Other limitations of using FC include difficulties in diagnosis of T-cell lymphoma and the fact that it is not helpful in the diagnosis of Hodgkin's lymphoma<sup>16,17</sup>. No false positive or negative results were encountered in our study, and although on limited number of cases, these results indicate that EUS-FNAC with FC is quite a sensitive and specific tool in the diagnosis of deep-seated

B-cell non-Hodgkin's lymphomas. Cytologic diagnosis combined with flow cytometry can be performed in ma-

jority of cases, and may eliminate need for open biopsy in some cases, especially in relapsed disease.

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## ANALIZA KLONALNOSTI PROTOČNIM CITOMETROM PUNKTATA ČVOROVA DOBIVENIH UZ POMOĆ ENDOSKOPSKOG ULTRAZVUKA

### SAŽETAK

Imunofenotipizacija protočnom citometrijom ima važnu ulogu pri postavljanju dijagnoze ne-Hodgkinovih limfoma (NHL) iz citoloških punktata. Analiza klonalnosti lakih lanaca imunoglobulina predstavlja najčešće korištenu metodu za potvrdu dijagnoze B-staničnih limfoma. Cilj ovog rada bila je procjena vrijednosti citoloških punktata teško dostupnih limfnih čvorova dobivenih uz pomoć endoskopskog ultrazvuka (EUS-a) za analizu klonalnosti protočnim citometrom te usporedba dobivenih rezultata s morfološkom dijagnozom. Nakon brzog bojanja i analize jednog od direktnih citoloških razmaza punktata, u slučaju postavljene sumnje na limfom, dodatni uzorci su poslani na analizu protočnim citometrom. Na analizu klonalnosti protočnim citometrom koristeći kappa/lambda/CD19/CD45 monoklonska protutijela primljeni su uzorci od šesnaest bolesnika. Analiza je izvršena na 11 uzoraka. Monoklonalnost je dokazana u 7 od 11 slučajeva citološki dijagnosticiranih NHL-ova, a 4 uzorka citološki dijagnosticirana kao benigna bila su poliklonalna. Naši rezultati, iako na manjem broju uzoraka, pokazuju da je citološka analiza punktata dobivenih punkcijom vođenom EUS-om uz protočnu citometriju dovoljno osjetljiva i specifična metoda za ocjenu limfadenopatije te postoji mogućnost da se ponekad izbjegne otvorena biopsija teško dostupnih čvorova, posebno kod relapsa bolesti.