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(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

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**Keywords:** flow cytometry; fine-needle aspiration cytology; thyroid lymphomas

## **Abstract**

### **Background**

To evaluate the efficacy of the use of flow cytometry (FC) immunophenotyping together with fine-needle aspiration cytology (FNAC) in the diagnosis of thyroid lymphoma.

### **Methods**

FC was performed in parallel with FNAC in 35 samples of suspected thyroid lymphoma over a 12 years period. Results were correlated with histological or molecular findings and follow-up, when available.

### **Results**

A final diagnosis of lymphoma was given in 13 of 35 (37.1%) specimens. Among the 22 cases considered negative for lymphoma by FC, 11 were diagnosed as thyroiditis by cytology, 7 as reactive, 2 were anaplastic carcinoma, and 2 cases were considered cytologically suspicious for lymphoma but were not confirmed by further investigations. Histology on core biopsy or molecular analysis was available in 12 of 13 lymphoma cases (92.3%). Data obtained by the combination cytology/FC were confirmed in all cases on histology biopsies. Correlation with histology showed a sensitivity and a specificity of 100% for the combination cytology/FC.

### **Conclusions**

FC is an important additional test that can contribute with cytology to the identification of lymphomas of the thyroid. FC can detect the presence of small neoplastic lymphocyte populations and may contribute to the diagnosis of cases in which the lymphoid infiltrate is difficult to interpret on cytology alone. © 2014 International Clinical Cytometry Society

Primary thyroid lymphoma (TL) is a rare and heterogeneous disease comprising 1 to 5% of all thyroid malignancies and 1 to 2.5% of all extranodal lymphomas. It includes several lymphoma subtypes <sup>1</sup>. About 80% of cases are B-cell non-Hodgkin's lymphoma (NHL), arising in a background of chronic lymphocytic thyroiditis (Hashimoto thyroiditis, HT) <sup>2</sup>. HT results from chronic antigenic stimulation and induces the clonal selection of B-lymphocytes and lymphoma development in many

cases<sup>3</sup>. The most frequent type is diffuse large B-cell lymphoma (DLBCL, about 70% of cases) with an aggressive clinical course. Second in frequency is the mucosa associated lymphoid tissue (MALT) lymphoma (approximately 30% of cases) which has a relatively indolent course. Hodgkin's lymphoma and rare cases of lymphomas of T-cell origin have been also reported in the literature<sup>2, 4</sup>. The diagnosis of the thyroid lesions is usually performed by fine-needle aspiration cytology (FNAC) which avoid open biopsy or lobectomy and allows for tailored treatments that may improve survival<sup>2, 5</sup>. Because of histopathological overlaps, the distinction between TL and lymphocytic thyroiditis may represent a challenging diagnostic dilemma for cytopathologists<sup>6</sup>. Several articles have reported the utility of flow cytometry (FC) immunophenotyping in conjunction with FNAC in the identification of lymphoid neoplastic cells<sup>7-10</sup>, but only few reported the use of FC in thyroid cytopathology<sup>11, 12</sup>.

In this article we report the collaborative multidisciplinary experience of a group of radiologists, surgeons, cytopathologists, and flow cytometrists in the diagnosis and classification of TL over a 12 years period. We describe the usefulness of an integration between these approaches and, in particular, we highlight the utility of FC in contributing to the diagnosis of cytologically incertain cases.

## MATERIALS AND METHODS

### Case Selection

The pathology database of our Institution was searched for all thyroid FNAC between 2001 and 2013 (7,865 cases). A total of 35 patients with both FNAC and FC were retrieved for this retrospective study. Data about age, gender, presenting symptoms, final diagnosis, therapy, and follow-up were collected and reviewed.

### Cytology Evaluation

A first aspiration was performed using a 22-gauge needle by a radiologist or a surgeon under ultrasound guidance. Two slides were immediately smeared by a cytopathologist, one fixed with 95° alcohol for 5 min and stained with hematoxylin and eosin (H&E) for rapid onsite evaluation (ROSE) in order to check the adequacy of the specimen<sup>13</sup>, the other was air dried for Giemsa staining (for diagnostic purpose). The remaining cytological material was rinsed in a tube containing ethanol for cell-block preparation: serial sections of the cell-block were cut and stained with H&E and the Papanicolaou method. When after ROSE, the smears showed cellularity with

a high number of lymphocytes, a second aspiration with fine needle was performed for FC and the cells were rinsed in a tube with RPMI with 10% fetal calf serum (hold medium). The FNACs were reported using standard cytological criteria, according to the Bethesda System For Reporting Thyroid Cytopathology <sup>14</sup>. Whenever needed, immunocytochemistry (ICC) was performed on serial paraffin-sections of the cell-block. Immunostaining with an avidin-biotin complex immunoperoxidase method was carried out using an automated immunostainer (BenchMark XT, Ventana Medical Systems, Tucson, AZ). The antibody panel included CD3 (clone PS1, Novocastra, Newcastle upon Tyne, UK), CD20 (clone L26, DAKO, Carpinteria, CA), and other stains (e.g. cytokeratin for epithelial cells) when appropriate. Positive and negative controls were performed in parallel for each assay. The specimens were analyzed based on the basis of pattern of the predominant cell population and were grouped as predominant large-cells, small-cells, mixed small, and large cells.

### **Flow Cytometry**

Samples for FC, kept in hold medium, were immediately sent to the FC laboratory and stained within 2 h from collection. Viability assessment was determined manually by trypan blue or by FC with the fluorescent dye 7AAD; all the detailed procedure has been previously reported <sup>8</sup>. Briefly, the samples were lysed with ammonium chloride to remove red blood cells when necessary, than aliquots (usually 100 µl) were directly labeled by multicolour immunofluorescence with four, six, or eight fluorochrome conjugated monoclonal antibody (MoAb) cocktails. The following fluorochromes were utilized: FITC (fluorescein isothiocyanate), PE (phycoerytrin), PerCP (peridinin chlorophyll protein) or PerCP-cy5.5 (peridinin chlorophyll protein-cyanin5.5), PE-cy7 (phycoerytrin-cyanin7), APC (allophycocyanin), APC-H7 (allophycocyanin-H7), HV450 (Horizon V450), HV500 (Horizon V500). Fluorochrome combination varied over time, as related on instruments configuration which was in use. We always used a monoclonal antibody panel for the screening and an additional panel in the case of lymphoma, if sufficient material was available (Table <sup>1</sup>). The proliferation value was assessed by FC evaluating Ki-67 on all cells, on large cells when clearly distinguishable from the small ones <sup>15</sup>. Data acquisition was performed on a FACS-Calibur or FACS-Canto II flow cytometer (Becton Dickinson, San Jose, CA) using CellQuestPro or Diva softwares for the analysis. A minimum of 20,000 events was collected for each sample. Sample analysis was first performed on forward-angle light scatter (FSC) versus right-angle light scatter (SSC) or on CD45 bright/low SSC population to identify lymphocytes;

multiple gating strategies were then utilized to define neoplastic population as CD19 or CD20 versus FSC or SSC, or CD3 versus FSC or SSC, or any other useful parameter. A small cell population was defined as complete overlap of the neoplastic population with the T-cell population on forward scatter histogram. A large cell population was defined as a complete shift of the neoplastic population from the T-cell population without overlap. If the neoplastic population exhibited a significant shift but substantial overlap with the T-cell population, the cells were defined as intermediate. A pattern of “normality” was established based on the presence of a mixture of T and B cells without evidence of monoclonality or aberrant immunophenotype. For B-cell data, the normal range of  $\kappa/\lambda$  ratio was previously determined in the laboratory and monoclonality was considered present if the  $\kappa/\lambda$  ratio was greater than 3:1 or less than 1:2. In selected cases, B-cell clonality was determined by the quantification of  $\kappa$  and  $\lambda$  chains on CD10 positive B-cells or on B-subset identified by bright or low B-cell antigen expression, or different FSC or SSC parameters. An abnormal pattern of CD4 or CD8 expression (e.g., aberrantly low or high, coexpression of both, lack of them), lack of expected T-cell markers (e.g. CD2, CD3, CD5, CD7), or otherwise aberrant staining intensity for any T-cell marker was considered indicative for T-cell lymphoma.

**Table 1. Monoclonal Antibodies used for Flow Cytometry Investigations**

Screening panel ( <i>clone, source</i> )	sCD3 (SK7, BD), CD4 (SK3, BD), CD5 (BL1A, Coulter), CD8 (SK1, BD), CD10 (MEM 78, Caltag), CD56 (B159, BD), CD19 (SJ25C1, BD), CD20 (B9E9, BD), CD30 (BER-H2, Dako), CD45 (2D1, BD), $\kappa$ (TB28-2, BD or polyclonal Dako), $\lambda$ (I-155-2, BD or polyclonal Dako)
Additional panel in case of B-cell lymphoma	CD11c (D12, BD), CD22 (B-ly8, IQP), CD23 (EBVC5-5, BD), CD25 (ACT-1, Dako), CD38 (HB7, BD), CD43 (DFT1, Coulter), CD79b (CB3-1, Southern B), CD103 (Bly-7, IQP), CD200 (MRC OX-104, BD), IgA - IgG - IgD - IgM (polyclonal, Biosource), FMC7 (FMC7, BD), Bcl-2 (124, Dako), Ki-67 (MIB1, Dako)
Additional panel in case of T-cell lymphoma	CD1a (VIT6B, Caltag), CD2 (S5.2, BD), CD7 (CD7-6B7, Caltag), CD16 (3G8, Caltag), CD57 (TB01, Dako), TCR $\alpha$ - $\beta$ (WT31, BD), TCR $\gamma$ - $\delta$ (11F2, BD), TCR V $\beta$ repertoire (IOTest kit, Coulter), cyCD3 (UCHT1, Dako)

s, surface; cy, cytoplasmic; BD, Becton Dickinson San Jose, CA; Coulter Immunology, Hialeah, FL; Caltag, Burlingame, CA; Biosource, Camarillo, CA, Dako; Glostrup, Denmark; Southern Biotechnologies, Birmingham, UK.

## **Molecular Studies**

Gene rearrangement studies for B- or T-cell clonality were analyzed by polymerase chain reaction (PCR) in selected cases if sufficient material was available, as previously described<sup>16</sup>.

## **Statistical Analysis**

The sensitivity, specificity, positive, and negative predictive values for the combination cytomorphology/FC were calculated in cases having a definite histologic diagnosis.

## **RESULTS**

The 35 FNAC specimens were from 35 patients (10 males and 25 females) with age ranging from 33 to 89 years (median age, 66 years). All the patients presented a diffuse palpable enlargement of the thyroid gland and single or multiple nodules sonographically detectable. All the cases selected had a highly cellular smear predominantly composed of lymphocytes at the onsite evaluation and a clinical history of lymphoma in one case. ICC on cell block sections showed a diffuse CD20 positivity in 14 of 35 cases and a prevalence of CD3 cells in 1 case. The FNAC evaluation was first performed blindly of the FC results and cases were classified as follows: positive for NHL when a concordant lymphoma diagnosis was obtained by FNAC and FC (FNAC+ and FC+), negative for NHL (FNAC- and FC-) or suspicious (s) for NHL if a diagnosis of lymphoma was suspected but not undoubtedly proven. Eleven of 35 specimens (31.4%) were considered positive for NHL (FNAC+/FC+) (Table 2). A subsequent core biopsy was performed in 9 of 10 (90%) FNAC+ and FC+ cases and all were confirmed by histology. The case identified as precursor T-lymphoblastic lymphoma (PTLL) by FNAC and FC was confirmed by a positive TCR rearrangement. Two cases that were considered suspicious for NHL by cytology and positive by FC (FNACs/FC+) were both confirmed as NHL by the subsequent histological biopsy.

**Table 2. Summary of the Overall Results**

	No. cases	Cytologic/flow cytometric conclusion
FNAC+/FC+	11	10 B-NHL; 1 PTLL
FNACs/FC+	2	2 B-NHL
FNAC-/FC-	20	11 thyroiditis; 7 reactive; 2 carcinoma
FNACs/FC-	2	2 reactive
Total	35	

FNAC: cytomorphology negative for lymphoma; FNAC+: cytomorphology positive for lymphoma; FNACs: cytomorphology suspicious for lymphoma; FC-: flow cytometry negative for lymphoma; FC+: flow cytometry positive for lymphoma; B-NHL: B-cell non-Hodgkin lymphoma; PTLL: precursor T-lymphoblastic lymphoma.

Among cases considered negative for lymphoma (FNAC-/FC-), 11 were diagnosed as thyroiditis, 7 as reactive, and 2 were anaplastic carcinoma with an inflammatory component by cytology. Three cases, two thyroiditis and one anaplastic carcinoma, were confirmed by histology.

Two cases were suspected to be lymphoma by cytology but were negative according to FC evaluation (FNACs/FC-); in these cases, molecular biology was not able to demonstrate clonality, thus failing to demonstrate a NHL. No cases with discordant FNAC+/FC- were detected.

The sensitivity, specificity, positive and negative predictive values for the final diagnoses obtained by combining cytomorphology with FC in the 12 cases having histologic or molecular follow-up were of 100% respectively.

### **Specific FC Findings**

Among samples detected as positive for lymphomas by FC, 12 cases were of B cells and one was of T cells (Table 3). In B-NHLs, the B-cell clonal population ranged from 6% to 75% of the overall lymphocyte population analyzed. In eight cases (no.1, 2, 5, 6, 7, 9, 12, 13) almost all B cells belonged to the neoplastic clone; in four cases <sup>3, 4, 10, 11</sup> malignant B cells were admixed to normal polyclonal B cells and the overall  $\kappa/\lambda$

ratio was normal. Monoclonal B lymphocytes were detectable only by gating on CD19 or CD20 brighter cells or on slightly augmented FSC (Figs. 1 and 2). Seven cases displayed  $\kappa$  and three cases displayed  $\lambda$  light-chain restriction; two cases did not express surface immunoglobulins. Eight cases (no.1, 3, 4, 5, 9, 11, 12, 13) were CD19 and CD20 positive while CD5 and CD10 negative. Six of them (no.1, 3, 4, 5, 9, 11) were composed of small-to-medium B cells consistent with MALT lymphoma by FC and were confirmed by histology. Cases no. 12 and 13 were classified as DLBCL. In contrast four cases (no. 2, 6, 7, 10) expressed the CD10 antigen, suggestive of germinal center origin; case no. 2 coexpressed CD5 and CD10 and had a very high proliferation index, suggestive of DLBCL by FC. Two of these cases were confirmed as DLBCL in the subsequent biopsy; case no. 7 was histologically diagnosed as mixed MALT/DLBCL lymphoma; case no. 6 was a Burkitt lymphoma by histology and fluorescence *in situ* hybridization confirmed the presence of the IgH/c-myc translocation t<sup>8,14</sup>.

The PTLL case (no.8) was of CD3-positive lymphoblasts displaying an immature immunophenotype composed of CD1a, CD4, CD8, and TdT-positive cells. Molecular biology studies confirmed the presence of a T-cell receptor (TCR) rearrangement.

### Clinical Follow-Up

Among patients who had a FC result positive for lymphoma clinical follow-up was available in 11 cases (10 B-NHL, 1 T-NHL) (Table 3). All the patients were treated and received a standard therapeutic treatment (chemotherapy, local radiotherapy, or surgery) according to their lymphoma subtype <sup>2</sup>. Among B-NHL, nine patients are alive till date, six are in remission, one had progressive disease, one is currently on chemotherapy. The patient having the PTLL was treated at diagnosis and is now in complete remission, 4 years since diagnosis.

### DISCUSSION

Fine-needle aspiration is the procedure of choice for the initial evaluation of a thyroid nodule, as it reduces the number of unnecessary thyroid surgery in benign cases and appropriately triages patients with malignancies. Among the diseases affecting the thyroid, the identification of a TL may represent a challenging differential diagnosis for the cytopathologist due to the difficulty in distinguishing neoplastic from benign lymphocytes that infiltrate the thyroid gland. The differential diagnosis is particularly difficult between HT and low-grade MALT lymphoma as MALT is composed of a mixture of normal and neoplastic lymphoid elements and it may be difficult to distinguish from a reactive lymphoid infiltration, such as in florid

lymphocyte type of autoimmune thyroiditis with scant epithelial cells [1, 6]. In contrast, large cell lymphomas are more easily identified by the presence of large cells with abundant cytoplasm, multilobated nuclei, and nucleoli. The diagnosis of TL is critical as it is a disease with good prognosis without the need of extensive surgery<sup>2</sup>.

The pathogenesis of TL is only partially understood. It is well known that in HT the gland is colonized by B cells which are involved in the autoimmune process and their chronic proliferation can evolve into a clonal TL [5]. Almost 50% of patients with TL have a clinical history of HT [1]. Moreover, it has been demonstrated that TL may evolve from HT, as the same clonal bands in HT can be subsequently detected in TL [17]. The tight relationships between TL and HT strengthen the concept that the differential diagnosis cannot rely on cytological evaluation alone and ancillary studies (as ICC or FC for demonstration of monoclonality) are necessary in all these cases, as suggested by the National Cancer Institute<sup>18</sup>.

In this article, we report our experience in detecting TL by the simultaneous use of FC together with FNAC. To our knowledge, only few articles have reported the systematic use of FC in detecting TL<sup>11, 19-22</sup>. In this study, FC was utilized together with FNAC to improve the detection of lymphomas among lymphoid infiltration of the thyroid. When the cytopathologist observed a smear with a predominant lymphocyte population during ROSE, a second aspiration for FC was requested. FC was able to identify the presence of an altered immunophenotype in 13 samples: 12 received a final diagnosis of B-cell related lymphoproliferative disorder, 1 was diagnosed as PTLL.

The majority of B-lymphomas detected among our cases were of MALT-type. In MALT lymphomas, lymphoepitelial lesions are characterized by lymphocytes stuffing the glandular lumina, representing colonization of the thyroid follicles by neoplastic B cells, with frequent plasmacytic differentiation. As previously stated, the cytological diagnosis of MALT in the thyroid is often difficult if not impossible, due to heterogeneous appearance of the neoplastic infiltrate. This may be composed of monotonous small centrocyte-like population, but also of a spectrum of small to large lymphoid cells<sup>23</sup>. The lymphoid infiltrate might mimic the benign lymphocytes seen in HT. In this study, seven patients with a documented previous chronic lymphocytic thyroiditis posed this diagnostic dilemma. Cytologically, the smears were composed of round lymphoid cells with a small rim of cytoplasm. The chromatin pattern was irregular and clumped. Cell-block sections showed clusters of monotonous lymphoid cells that resulted diffusely positive for CD20 by ICC (Fig. 3). In four of these cases, FC did not show unbalanced  $\kappa/\lambda$  ratio among B cells, while in three cases a clonal B-cell population was detectable, suggesting the presence of lymphoma. The possibility to detect surface light chains together B-cell antigen

expression and to correlate antigen density with the lymphocyte-size, as offered by multiparameter FC analysis, helps in distinguish normal from neoplastic cells, making this methodology highly sensitive in detecting small neoplastic populations<sup>10, 15</sup>. Differences in staining intensity among B-cell antigens detected by FC may indeed be suggestive of lymphoma. It is well known that Follicular lymphoma may express reduced level of CD19<sup>24</sup>, but also an increased expression of B-cell markers is indicative of potential lymphoma. As illustrated in Figure 1, the FC analysis allowed in many cases the identification of clonally restricted B cells, hidden among polyclonal B cells, on the basis of their brighter CD19 expression coupled with a slightly augmented FSC parameter. In other cases, a clear FSC increase of the B population was the sign of the presence of DLBCL, suggested also by the absence of surface immunoglobulin expression (Fig. 4).

A skewed  $\kappa/\lambda$  ratio has been reported in HT<sup>12, 25, 26</sup> and caution has been suggested in considering the imbalanced  $\kappa/\lambda$  ratio as diagnostic of lymphoma<sup>27, 28</sup>. Prominent clonal B-cell populations have been described in reactive situations, particularly in cases in which follicle center derived B cells were involved, although they represent a rare finding<sup>29</sup>. An abnormal immune response to microbial antigens or autoimmune stimulation, leading to light chain restriction as the expression of somatic hypermutation, has been suggested in these cases<sup>10</sup>. The detection of small amount of clonally restricted B cells, mainly in patients with a previous history of thyroiditis, may really represent a diagnostic challenge. In our study, however, the three patients with a pre-existing HT that developed B-lymphoma, had increased B-cell numbers with all B cells belonging to the neoplastic clone in case 1, or clonally restricted B cells clearly distinguishable from normal polyclonal B-lymphocytes in cases 4 and 11. We think that the powerful capacity of the multiparameter FC analysis to carefully dissect an unbalanced  $\kappa/\lambda$  ratio is the best way to identify neoplastic populations.

The cytological diagnosis of DLBCL in the thyroid is considered to be simpler respect to MALT diagnosis, if a monomorphic population of large cells (two to three times the size of a mature lymphocyte), lack of cellular cohesion and lymphoglandular bodies in the background were seen. The distinction between DLBCL and anaplastic thyroid carcinoma can also be difficult by FNAC if cytologic features of B cell resembles pleomorphic feature of anaplastic carcinoma cells<sup>30, 31</sup>. Ancillary studies such as FC and ICC are necessary in these cases to perform a differential diagnosis. Among our patients, cases no. 10 and 13 had a monotonous infiltrate of small-to-medium lymphocytes with intermingled large cells with eccentric nucleus, unevenly

distributed chromatin, central distinct nucleoli. FC was able to identify a small fraction of medium to large CD10-positive B cells, that displayed surface κ light chain restriction together monotypical heavy chain (IgG type) expression in case no. 10 and a consistent amount of clonally restricted B cells in case no. 13. In two patients (cases no. 7, 12) FC analysis was unable to detect surface light chain immunoglobulin expression, but neoplastic large B cells were nonetheless clearly mixed with normal reactive B- and T-lymphocytes (Fig. 4). Although PCR studies were not possible, due to scarcity of material (as in the majority of samples), FC and cytomorphology findings were highly suggestive of DLBCL. The FC results prompted a subsequent histologic evaluation by core biopsy that eventually confirmed the presence of lymphoma in both cases.

A case of PTLL was identified by this study. This type of lymphoma of the thyroid is extremely rare: 21 cases have been reported in the literature to date to our knowledge and only 3 had a FC evaluation<sup>11, 32, 33</sup>. FC analysis together with gene rearrangement study shortened the time to reach the final diagnosis, allowing a prompt start of the treatment.

No false-negative cases were detected by FC in this study. The addition of FC to FNAC analysis has been demonstrated to produce high level of sensitivity and specificity in detecting lymphomas<sup>7, 34</sup>. By adding FC to FNAC we obtained a value of 100% in both sensitivity and specificity, thus confirming that FC improved the diagnostic efficacy of FNAC also in the thyroid.

Some authors reported that inadequate sampling is a limiting factor to perform FC in conjunction with FNAC<sup>35, 36</sup>. We did not have inadequate sampling for FC in this study. We have experienced that the multicolor FC analysis, performed by the latest generation of clinical flow cytometers (up to eight antigens simultaneously detected), allows us to obtain useful information also from samples with low cellularity, thus reducing the number of inadequate samples<sup>37</sup>.

In summary, we report the largest series of FNAC of the thyroid also investigated by FC to date. The achievement of a final diagnosis of TL in 13 cases was reached by the conjunct use of FNAC/FC. We imply that FC plays a crucial role not only in the diagnosis of TL by detecting neoplastic populations, but also can help cytomorphologist in the characterization and evolution of reactive infiltrates in the thyroid. We believe that through the collaboration among practitioners expert in

different fields it is possible to rapidly achieve a correct diagnosis in most if not all cases.

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