



Flow cytometry in the differential diagnostics of Hashimoto's thyroiditis and MALT lymphoma of the thyroid

Cytometria przepływowa w diagnostyce różnicowej choroby Hashimoto i chłoniaka MALT tarczycy

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Abstract

Introduction: A combination of traditional cytology methods with fluorescence activated cell sorting (FACS) analysis of fine-needle aspiration biopsy (FNAB) material is considered a powerful diagnostic tool in the differential diagnosis of thyroid lesions suspected of mucosa-associated lymphoid tissue lymphoma (MALT-L).

The aim of this study was to demonstrate the FACS-based diagnostic process of thyroid lesions in a clinical situation where ultrasound and cytological examinations did not allow differentiation between Hashimoto's thyroiditis (HT) and MALT-L.

Material and methods: The patients analysed in this study presented significantly different clinical courses of thyroid disease: quickly enlarging painless tumour of the thyroid right lobe in the first case, and chronic HT with palpable tumour in the thyroid isthmus in the second patient. Due to the suspicion of MALT-L resulting from indeterminate ultrasound and FNAB-cytology results, FNAB material was obtained from all the previously examined thyroid lesions and directly subjected to FACS assessment, encompassing κ/λ light chain restriction analysis, as well as measurements of B and T cell surface antigens.

Results: The FACS analysis of FNAB material obtained from our patients did not show any definite signs of light chain restriction. Although one of the samples showed a borderline value of κ/λ ratio ($\kappa/\lambda = 0.31$), further immunophenotyping confirmed clonal expansion in none of the examined thyroid regions. Histopathological findings documented the diagnosis of HT in both clinical cases.

Conclusion: We believe that FACS represents a useful and reliable complementary diagnostic measure in FNAB-based differential diagnosis of lymphoproliferative thyroid disorders. (*Endokrynol Pol* 2015; 66 (1): 73–78)

Key words: fine needle aspiration biopsy; fluorescence activated cell sorting; Hashimoto's thyroiditis; MALT

Streszczenie

Wstęp: Skojarzenie oceny cytologicznej oraz cytometrii przepływowej (FACS, *fluorescence activated cell sorting*) materiału uzyskanego podczas biopsji aspiracyjnej cienkoigłowej (BAC) jest uważane za niezwykle skuteczną metodę w diagnostyce różnicowej zmian tarczycy podejrzanych o obecność pozawęzłowego chłoniaka strefy brzożnej systemu MALT (MALT-L).

Celem pracy było zaprezentowanie opartego na FACS procesu diagnostycznego zmian ogniskowych tarczycy u chorych, u których badanie ultrasonograficzne i cytologiczne nie umożliwiło zróżnicowania przewlekłego zapalenia tarczycy (HT, *Hashimoto's thyroiditis*) od MALT-L.

Materiał i metody: Chorzy opisani w pracy charakteryzowali się całkowicie odmiennym przebiegiem klinicznym choroby tarczycy — ujawniającej się w pierwszym przypadku jako szybko powiększający się guz płata prawego, w drugim jako zmiana ogniskowa w cieśni u chorej z przewlekłym wywiadem HT. Ze względu na podejrzenie MALT-L postawione na podstawie badania cytologicznego oraz podejrzany wzorec ultrasonograficzny, przeprowadzono ponownie BAC wszystkich wcześniej ocenianych zmian tarczycy, a uzyskany materiał poddano bezpośrednio ocenie za pomocą FACS, obejmującej analizę restrykcji łańcuchów lekkich immunoglobulin κ/λ oraz antygenów powierzchniowych limfocytów T i B.

Wyniki: Analiza FACS materiału uzyskanego za pomocą BAC nie ujawniła definitywnych cech restrykcji łańcuchów lekkich. Pomimo granicznych wartości współczynnika κ/λ ($\kappa/\lambda = 0,31$) w jednej z próbek, dalsza analiza fenotypowa nie potwierdziła klonalnej ekspansji w żadnym z badanych obszarów tarczycy. Wyniki histopatologiczne potwierdziły diagnozę przewlekłego zapalenia tarczycy w obu przypadkach klinicznych.

Wnioski: Cytometria przepływowa jest badaniem wiarygodnie uzupełniającym ocenę cytologiczną w diagnostyce różnicowej przewlekłego zapalenia tarczycy i limfoproliferacyjnych chorób tarczycy. (*Endokrynol Pol* 2015; 66 (1): 73–78)

Słowa kluczowe: biopsja aspiracyjna cienkoigłowa; cytometria przepływowa; przewlekłe zapalenie tarczycy; MALT



Introduction

Flow cytometry or fluorescence activated cell sorting (FACS) procedure does not belong to diagnostic standards in thyroid diseases. However, in some clinical cases it can significantly facilitate the final diagnosis. A combination of traditional cytology methods with FACS analysis of fine-needle aspiration biopsy (FNAB) material is considered to be a powerful diagnostic tool in haematological malignancies [1, 2, 3–6]. Assessment of κ/λ light chain clonality belongs to the most important procedures allowing differentiation between polyclonal reactive processes and monoclonal lymphoma [7, 8]. This applies, for example, to clinical cases where difficulties appear in differentiation between florid lymphocytic infiltration and non-Hodgkin's lymphoma (NHL) of the thyroid [9]. Moreover it is a particularly important element in the diagnosis of mucosa-associated lymphoid tissue (MALT) lymphomas (MALT-L) [10]. Cytologically, this type of lymphoma is characterised by a very heterogeneous appearance of neoplastic infiltration [6]. In consequence, the traditional cytologic examination of FNAB material has been proven to be insufficient in the differential diagnosis of thyroid lesions suspected of MALT-L [11, 12]. In contrast to NHL, no clear literature data exists regarding the applicability of FACS analysis of FNAB material in other rare lymphoproliferative processes of the thyroid, such as Hodgkin's lymphoma.

Thyroid lymphoma is a rare disease, comprising approximately 2% of all thyroid malignancies [13]. Primary involvement of the thyroid is found in about 2.5% of all extranodal lymphomas [14]. Most thyroid lymphomas are B-cell lymphomas: more than 50% of cases are diffuse large B-cell lymphoma (DLBCL), and 10–23 % of cases are extranodal marginal zone B-cell lymphomas, also known as MALT. Other subtypes of thyroid lymphomas include: follicular (10%), small lymphocytic (3%), and Hodgkin's lymphoma (2%). Burkitt's, T-cell, mantle cell and lymphoblastic lymphomas each account for less than 1% of cases [15–17].

The clinical picture of thyroid lymphoma is frequently associated with a rapidly enlarging painless thyroid mass, leading to a compression of neck structures with cervical lymph-node enlargement. Many patients complain of unexplained exhaustion, dryness of mucosal surfaces, loss of weight, fever and night sweats — symptoms especially prominent in DLBCL patients [18].

Usually in such cases the diagnosis is not difficult. However, in some patients the thyroid tumours have an indolent course without clinical symptoms suggestive of an aggressive lymphoma. Such a pattern of disease usually occurs in MALT-L [19–21]. This NHL subtype

develops outside lymph nodes (extranodal expansion). The cells of MALT-L typically express pan-B-cell markers, e.g. CD19 and CD20 and are negative for CD10 and CD5, although some cases of CD5 positive MALT-L have been reported [22]. Most MALT-L cases begin in the gastrointestinal tract, especially in the stomach. Other possible initial sites of MALT-L include the lungs, skin, salivary glands, ocular adnexa and the thyroid. In many cases, the development of MALT-L was linked to infectious factors (such as *Helicobacter pylori* in stomach, *Chlamydia psittaci* in ocular adnexa, *Borrelia burgdorferi* in skin) regarded as a primary source of chronic inflammatory reaction, leading in consequence to malignant transformation. Also autoimmune disorders associated with persistent immune processes in target organs (e.g. salivary gland in Sjögren syndrome) are considered as factors increasing the risk of developing MALT lymphoma. In the thyroid, a positive correlation of a long-term chronic autoimmune thyroiditis (such as Hashimoto's thyroiditis, HT) and the risk of MALT-L have been suggested [23]. In the light of the aforementioned etiopathological findings, it is considered that the first line of treatment should be cessation of the irritating factor such as *Helicobacter pylori* or *Chlamydia psittaci* in gastrointestinal or conjunctival/orbital MALT lymphoma, respectively. In the case of the thyroid, such a scheme is, for obvious reasons, not feasible.

Thyroid tissue is normally devoid of lymphocytic populations. In HT, progressive loss of thyrocytes is typically associated with an increasing grade of lymphoid infiltrates and the formation of germinal centres. The atrophic form of HT is characteristic for most patients but in some cases, or in particular phases of thyroiditis, unilateral or bilateral thyroid gland enlargement with the formation of nodules is observed. In such a clinical situation, the differentiation between inflammation and cancer represents often a serious diagnostic problem [24].

Material and methods

Here we present two patients with different clinical courses of thyroid disease. In both cases, diagnostic measures based on ultrasound of the neck and FNAB of the lesions were insufficient for full characterisation of the thyroid disorder. The diagnostic challenge was an inability to differentiate between MALT-L and HT [19].

Patient 1

A 50-year-old woman was admitted to the Department of Endocrinology and Metabolic Diseases due to a quickly enlarging painless tumour of the thyroid right lobe. A physical examination revealed the presence of a firm right lobe tumour with a diameter of

4 cm. The left lobe was impalpable. Laboratory tests revealed normal serum concentrations of free thyroid hormone fractions (fT3 and fT4) and thyrotropin (TSH) and increased serum levels of anti-TPO and anti-Tg antibodies. Serum concentration of anti-TSH receptor antibody (TRAK) was normal. Due to the rapid, painless volume increase of the right thyroid lobe paralleled by increased serum concentrations of anti-TPO and anti-Tg, diagnoses of anaplastic thyroid cancer or lymphoma were considered. Ultrasound examination demonstrated an asymmetry of the thyroid lobes: diameters of right — 30 × 37 × 58 mm; left — 17 × 19 × 41 mm. The entire right lobe had solid, heterogeneous, hypoechoic structure with decreased blood flow (Power Doppler). The margin was blurred and poorly defined without a halo. In the left lobe a single focal lesion (10 × 13 × 15 mm) with a very similar ultrasound pattern was revealed. The ultrasound structure of the tissue surrounding left lobe lesion was normoechoic and homogeneous. In both lesions, the risk score of malignancy was assessed as intermediate on the basis of ultrasound characteristics (4 points in the right and 4.5 points in the left thyroid lobe) [25]. The patient underwent FNAB of both lobes — the dominant lesion in the right lobe and the lesion in the left lobe. Cytological examination of the right lobe lesion revealed equivocal results, suggestive of MALT-L or HT. In the left lobe, cytology findings were consistent with HT characteristics.

Patient 2

A 46-year-old woman was seen in the endocrinology department due to Hashimoto's disease. The patient received LT₄ supplementation (125 mcg per day). A physical examination revealed the presence of a tumour in the isthmus with a diameter of 2 cm. Both lobes of the thyroid were characterised by a hard, uneven surface, without palpable nodules. Laboratory tests revealed normal serum concentrations of free thyroid hormones (fT3 and fT4) and TSH and increased serum levels of anti-TPO and anti-Tg antibodies. Concentration of TRAK was normal. Ultrasound examination demonstrated hypoechoic, heterogeneous structure of both thyroid lobes with decreased blood flow (Power Doppler): diameters — 18 × 17 × 48 mm; left — 14 × 15 × 50 mm of the right and left thyroid lobe, respectively. In the border between right lobe and isthmus, ultrasound revealed a focal, solid, hypoechoic lesion (19 × 15 × 17 mm) with hyperechoic spots (probably microcalcifications). The blood flow was decreased (Power Doppler) and the margin was blurred and poorly defined without a halo. The malignancy risk of the lesion was assessed as intermediate on the basis of ultrasound

pattern — 4.5 points [25]. The patient underwent FNAB of both lobes and lesion in isthmus. Cytological examination revealed HT in both lobes, whereas the lesion in the isthmus proved to be cytologically indeterminate, with a suspicion of MALT-L or HT.

Despite obvious differences in the clinical course, the results of FNAB examination in both cases were very similar and suggested the need for further differentiation between MALT-L and HT.

Therapy in patients with MALT-L appearing as thyroid tumour encompasses radiotherapy in the case of localised disease and chemotherapy in disseminated cases [20]. These therapeutic procedures differ substantially from the usual surgical treatment in patients with thyroid tumours derived from epithelial cells. For that reason, it is of crucial importance to make the correct diagnosis prior to therapy implementation.

In order to specify the diagnosis, both patients were subjected to another FNAB. Cellular material was obtained from thyroid regions examined previously with cytology techniques and subjected to FACS examination. For this purpose, a separate needle pass was performed in each assessed thyroid region and the obtained material was washed and suspended in 1 ml of PBS.

Patient 1 underwent FNAB of the right lobe lesion (sample 1A), the left lobe lesion (sample 1B) and the surrounding tissue of the left lobe, without ultrasound features of HT (sample 1C) (Fig. 1).

Patient 2 underwent FNAB of both lobes - left lobe (sample 2A), right lobe (sample 2B) and of the lesion close to isthmus (sample 2C) (Fig. 2).

Flow cytometry was performed using a multicolour analysis technique on a FACSCanto II Cytometer (BD Biosciences, San Jose, CA, USA). The cells obtained with FNAB were incubated with a combination of phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and fluorescein isothiocyanate (FITC) conjugated antibodies specific for particular antigens: CD3+, CD5+, CD8+, CD10+, CD19+, CD20+, CD22+, CD38+, and κ and λ light chains. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). A population of 10,000 cells was considered sufficient for phenotypic analysis and the expression of each antigen was quantified as a percentage of the whole population with FACSDiva software (BD Biosciences, San Jose, CA, USA). The diagnostic criteria of MALT B-cell lymphoma were based on the evaluation of immunoglobulin light chain restriction in combination with an assessment of surface molecule expression pattern characteristic for a clonal population of B-lymphocytes [14, 26].

Light chain evaluation is regarded as a crucial step in the distinction between monoclonal (neoplastic) and polyclonal (reactive) B cell expansion. The κ/λ

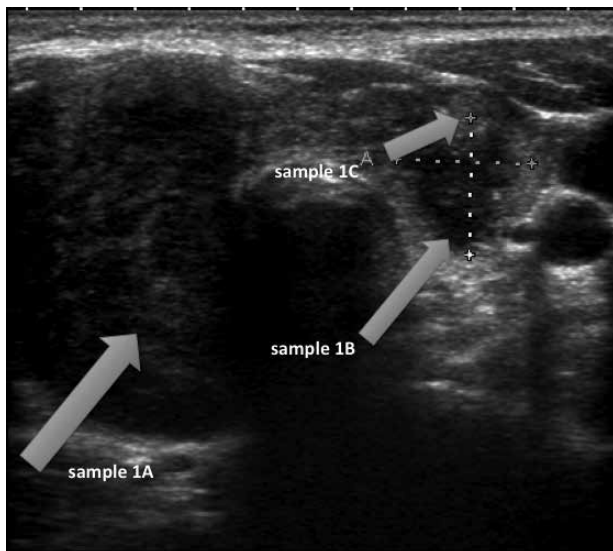


Figure 1. Ultrasound image of thyroid gland of Patient 1. Arrows indicate thyroid regions subjected to FNAB. Sample 1A — solid, heterogeneous, hypoechogenic structure of right lobe; sample 1B — solid, heterogeneous, hypoechogenic structure of 'taller-than-wide' focal lesion in left thyroid lobe with poorly defined margin, without a halo; sample 1C — normoechogenic structure of tissue surrounding left lobe lesion

Rycina 1. Pacjent 1 — miejsca aspiracji próbek. Próbkę 1A — lita, niejednorodna, hipoechogeniczna struktura płata prawego, próbkę 1B — lita, niejednorodna, hipoechogeniczna struktura "wyższej niż szerszej" zmiany ogniskowej płata lewego o zatartym brzegu bez otoczki typu „halo”; próbkę 1C — normoechogeniczna struktura tkanki otaczającej zmianę w lewym płacie

ratio in the 0.5–3.0 range is considered as typical for benign processes [7]. Accordingly, many authors have proposed κ/λ ratio > 3 –4.0 or λ/κ ratio $> 2:1$ as confirming monoclonality in the diagnosis of lymphoma [2, 27, 28]. However, clonal bands have been also reported in patients with a classical course of HT. Due to these observations, some authors have suggested more strict criteria of B cell clonal expansion with κ/λ ratios > 6.0 (> 10.0) or < 0.3 as definite evidence of monoclonality [29–32]. In cases of unclear monoclonality or suspected loss of light chain expression (cumulative number of κ and λ expressing cells does not approximate the number of B cells), further immunophenotyping with B- and T-cell markers has been indicated as a very helpful tool in precise lymphoma diagnosis and characterisation [33].

Results

Taking into consideration the abovementioned studies, in our analysis we adapted an algorithm proposed by Zardawi et al. for the FACS based analysis of FNAB material in the diagnosis of lymphoma [2], with modifications necessary for the diagnosis of le-

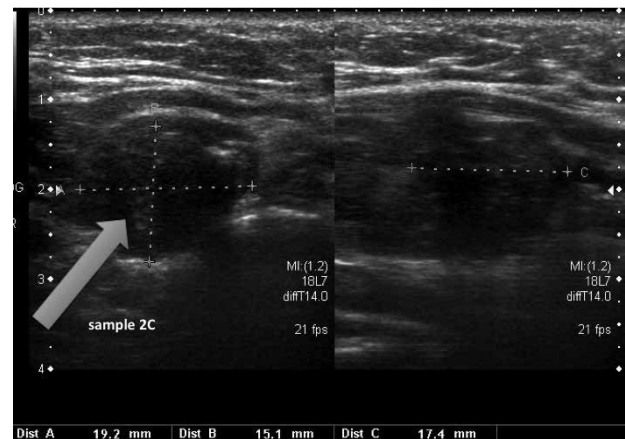


Figure 2. Ultrasound image of thyroid gland of Patient 2. Arrow indicates thyroid region subjected to FNAB (sample 2C) — solid, heterogeneous, hypoechogenic structure of focal lesion close to isthmus with hyperechogenic spots (probably microcalcifications) and poorly defined margin, without a halo

Rycina 2. Pacjent 2 — miejsce aspiracji próbki 2C — lita, niejednorodna, hipoechogeniczna struktura zmiany ogniskowej okolicy cieśni z hiperechogenicznymi punktami (prawdopodobnie mikrozwapnieniami) i zatartym brzegiem bez otoczki typu „halo”

sions in extranodal localisations. In order to properly differentiate from HT immune infiltrates, light chain restriction criteria were set as κ/λ ratio > 6.0 or < 0.3 . In the absence of light chain restriction, CD20 positivity exceeding 85% of the whole cellular fraction was set as confirmation of B-cell lymphoma. CD5 and CD10 molecules served as further characteristics of B cell lymphoproliferative processes characteristic for extranodal localisation (e.g. MALT-L). Of note, co-expression of both B and T cells antigens was regarded as very characteristic for HT [18].

The FACS analysis of FNAB material obtained from our patients did not show any definite signs of light chain restriction in the examined thyroid lesions (Tables I and II). Nonetheless, in the sample 2B, representing a cytologically HT picture, κ/λ ratio assessment resulted in a borderline value ($\kappa/\lambda = 0.31$, Table II). However, further immunophenotyping confirmed clonal expansion of B cells in none of the examined thyroid regions (including lesion 2B) (Tables I and II). The predominance of cells expressing B-cell antigens (CD19 and CD20) over T cells (CD3 positive cells) was only observed in sample 1C, representing material from the thyroid region with normal ultrasound structure (the area surrounding the focal lesion in the left lobe of patient 1). Interestingly, sample 1C was also characterised by the lowest percentage of lymphocytes compared to other FNAB samples from patient 1 (16.55 vs. 42.5% and 43.34%) and samples obtained from patient 2 where lymphocytes exceeded 70% of cellular FNAB fraction in all examined regions (Tables I and II).

Table I. Patient 1 data — results of flow cytometry and cytological examinations of FNAB specimens from the right thyroid lobe (sample 1A), left thyroid lobe focal lesion (sample 1B) and the area surrounding the focal lesion in the left lobe (sample 1C)

Tabela I. Dane pacjenta 1 — wyniki cytometrii przepływowowej i badania cytologicznego próbek pobranych za pomocą BACC z płata prawego tarczycy (próbka 1A), zmiany ogniskowej płata lewego tarczycy (próbka 1B) oraz wynik cytometrii przepływowowej próbki pobranej z obszaru otaczającego zmianę ogniskową w płacie lewym (próbka 1C)

FNAB result	MALT or HT	HT	–
	Sample 1A Lymphocytes 42.50%	Sample 1B Lymphocytes 43.34%	Sample 1C Lymphocytes 16.55%
CD19	35.15	16.87	79.15
CD20	35.24	17.51	66.83
CD5	31.41	81.99	17.89
CD3	58.98	80.28	29.2
κ/λ ratio	1.92	1.42	0.93

Discussion

These findings demonstrate that the morphological lesions in thyroid gland observed in US may correlate with the intensity and quality of immune infiltration. The differences between patients may be associated with different phases of disease activity, as well as with the level of concomitant fibrosis (e.g. patient 1), which found its confirmation in histopathology examination (see below). Furthermore, various immunophenotypes of infiltrates in particular thyroid foci in the same patient may also suggest an existence of different types of local immune reactions. Such an assumption concurs with the study by Zeppa et al. who described two main immunophenotypes occurring in HT: B-cell lesions with a significant presence of CD19 positive cells ($\geq 20\%$) and T-cell lesions with a prevalence of CD3, CD5 expressing cells and CD19 positive cells not exceeding 20% of cellular fraction. According to these authors, there is a relationship between clinical symmetrical enlargement and T-cell phenotype as well as between nodular presentation and B-cell phenotype [9]. According to this qualification, in our study Sample 1B was characterised by T-cell pattern, whereas samples 1A, 2A, 2B and 2C represented B-cell pattern.

Unconfirmed MALT-L diagnosis and the intermediate ultrasound risk score of malignancy were the reasons for qualification of both our patients to surgery. Histopathological findings clearly ruled out the diagnosis of lymphoma of the thyroid. In Case 1, chronic thyroiditis with intensive fibrosis features was found. In Case 2, chronic thyroiditis was diagnosed. In this

Table II. Patient 2 — results of flow cytometry and cytological examinations of FNAB specimens from the left thyroid lobe (sample 2A), right thyroid lobe (sample 2B) and lesion in the isthmus (sample 2C)

Tabela II. Dane pacjenta 2 — wyniki cytometrii przepływowowej i badania cytologicznego próbek pobranych za pomocą BACC z płata lewego tarczycy (próbka 2A), płata prawego tarczycy (próbka 2B) i ze zmiany ogniskowej w cieśni (próbka 1C)

FNAB result	MALT or HT	HT	HT
	Sample 2A Lymphocytes 74.00%	Sample 2B Lymphocytes 76.00%	Sample 2C Lymphocytes 75.00%
CD19	21.2	42.9	36.5
CD20	19.8	44.3	34.3
CD5	68.5	55.2	61.8
CD3	71.0	55.8	61.2
κ/λ ratio	0.67	0.31	1.16

context, there were surprising differences in the clinical course: a very quickly enlarging tumour characterised euthyroid patient 1, whereas indolent behaviour characterised hypothyroid patient 2.

Taken together, on the basis of the presented two clinical cases, we believe that FACS can be a very useful and reliable complementary diagnostic measure in FNAB based differential diagnosis of lymphoproliferative thyroid disorders. However, further studies including larger groups of patients are required to define exact clinical applications. Moreover, implementation of this method requires effective co-operation between clinicians and pathologists specialist both in haematology and in flow cytometry performance and interpretation.

Acknowledgement

This study was financially supported by the Medical University of Lodz (503/1-107-03/503-01).

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