Sheremet M. I., Shidlovskyy V. O., Sydorchuk L. P. Assessment of proliferation and apoptosis markers in patients with autoimmune thyroiditis. Journal of Education, Health and Sport. 2016;6(1):179-188. eISSN 2391-8306. DOI http://dx.doi.org/10.5281/zenodo.45327
http://ojs.ukw.edu.pl/index.php/johs/article/view/45327
https://pbn.nauka.gov.pl/works/709555

The journal has had 7 points in Ministry of Science and Higher Education parametric evaluation. Part B item 755 (23.12.2015).
755 Journal of Education, Health and Sport eISSN 2391-8306 7

The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 15.12.2015. Revised 12.01.2016. Accepted: 25.01.2016.

UDC 616.441-002: 616-002.18

ASSESSMENT OF PROLIFERATION AND APOPTOSIS MARKERS IN PATIENTS WITH AUTOIMMUNE THYROIDITIS

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Abstract

The paper presents results of a comparative analysis of apoptosis and proliferative activity in the tissue of the thyroid gland (TG) in patients with autoimmune thyroiditis (AIT) and thyroid adenoma (TA) compared with morphologically unaltered thyroid tissue. In immunohistochemical study of the tissue we detected high proliferative activity of lymphoid tissue in patients with AIT, moderate proliferative activity of tissue in TA and low activity beyond it. In AIT one can notice intensive expression of Fas, Bcl-2 and Ki-67 compared to both morphologically unchanged tissue and the thyroid adenoma, which indicates immunological destruction of the thyroid epithelium. While studying combined indicators of apoptosis and proliferation in these patients we found out that in patients with AIT the number of all groups of connected cells is by 3-25 times higher as compared both to the group of patients with TA and to those with morphologically unchanged thyroid tissue. We also discuss possible mechanisms of the disorders found.

Key words: thyroid gland, autoimmune thyroiditis, apoptosis, markers of proliferation.

Introduction. In Ukrainian medical literature there are many publications devoted to the study of the thyroid gland morphology in autoimmune thyroiditis. However, a number of unresolved issues remain, including the role of AIT in the development of cancer. According to some authors, in AIT we can observe some metaplasia of the thyroid epithelium, hyperplasia of lymphoid tissues, which certainly can be considered as a facultative precancerous condition [1].

Their information is confirmed by the fact that papillary cancer and limphomas in patients with AIT occur three times more frequently than in forms of nodular goiter [2, 3]. Other authors argue that anti-tumor immunity as lymphoplasmocytic infiltration should be regarded as AIT and AIT combined with thyroid tumors is rather rare [4 - 6].

The total accuracy of clinical, instrumental and laboratory diagnostic methods for establishing morphological origin of the thyroid nodular tumors even in the most daring conclusions does not exceed 80% [7]. Such a result can not satisfy either surgeons (unjustified over diagnosis of the thyroid cancer) or endocrinologists (inadequate and delayed selection of patients for a surgery). It is rather difficult to establish the malignancy of the node at early stages, and the diagnosis is made at later stages when there are regional and distant metastases [8, 9]. Numerous immunohistochemical, immunocytochemical and molecular markers, none of which, unfortunately, is wholly specific, are suggested as criteria for AIT and TG cancer differential diagnostics [5, 6, 8, 9].

One of the mechanisms of tumor transformation and progression is cell cycle dysregulation with apoptosis inhibition and cell proliferation activation [10].

Marker Ki-67is considered to be perspective. Antibodies Ki-67-tion recognize nuclear DNA bound protein that is present in the nuclei of cells in 01-, 8-, 02- and M-phases and which is absent in 0_0-phase. Proliferative activity of many tumors is evaluated using Ki-67. We found a connection between proliferation index, degree of histological differentiation of the tumor and clinical prognosis in malignant tumors of the endometrium, ovaries, lungs, breast, bladder, lymphoma and tumors of the nervous system [10-13].

Oncomarker p53 is also promising for diagnostic use. Protein p53 as a product of the tumor suppressor gene p53, is expressed in all cells of the body. The result of its activation is the cell cycle and DNA replication arrest , and in excessive stress signal a start of apoptosis occurs. Protein p53 becomes active in damages of the genetic apparatus, as well as with stimuli that could lead to such damage, or they are a signal of unfavorable condition of the cells (stress). The function of p53 is to remove the cells that are potentially oncogenic out of the pool, hence the figurative name of the p53 protein (guardian of the genome) - the keeper of the genome [13-17].

Several authors investigated the molecular markers of apoptosis and proliferation in malignant diseases of the thyroid and exophthalmic toxic goiter [4, 7, 8, 12, 13]. However, publications on the use of immunohistochemical markers for diagnosis and differential diagnosis of AIT are rare in the literature.

Thus, the ambiguous interpretation of results of studies by different authors allows concluding that the valuation of markers which regulate apoptosis and their relationships to each other (p53 protein and its mutations, bcl-2, Fas-system) and indicators of proliferation (Ki -67) during the transition from benign to malignant process and role in these autoimmune reactions are extremely topical issues.

Therefore, the use of immunohistochemical markers for differential diagnosis is promising not only in malignant thyroid tumors but for the prediction of autoimmune thyroid diseases course.

Objective

To study the apoptosis and proliferation activity and performance of cells' groups and receptors' density in / on cells with combinations of markers that regulate apoptosis and proliferation in AIT, TA tissue and in morphologically unchanged thyroid tissue sections using immunohistochemical method.

Material and methods

During 2013-2015 we have examined 75 women complaining about discomfort in the neck. We evaluated the hormonal status (TSH, free T4, free T3) ratio of antibodies to thyroglobulin (AB-TG) and to thyroid peroxidase (AT- TPD), the volume and structure of the thyroid gland (TG) according to ultrasound.

50 of them were diagnosed with AIT (I-group, the main). Indications for surgery in this group of patients were: enlargement of the thyroid gland with symptoms of compression and narrowing of the trachea and esophagus; the nodes compressed on the neck organs; progressive growth of goiter, despite ongoing for 1-1.5 years conservative therapy; suspected malignant degeneration, based on FNAB findings.

We selected a group of 25 women who, by ultrasound findings, by those of fine needle aspiration biopsy (FNAB) and histological conclusions after surgery were diagnosed with thyroid adenoma (II group). We have identified this group due to the fact that this pathology is one of the most common forms of nodular goiter.

Patients with TA were examined on their parenchyma of the contralateral part of the TG which had not been affected by the node and remained morphologically unchanged. These figures were used as control ones (group I-III). Final confirmation of morphologically unchanged tissue was obtained after histological conclusion.

The study did not involve patients with hyperthyroidism, clinical hypothyroidism,

hypertension and cardiovascular diseases, severe somatic pathology and those after the menopause onset.

All patients underwent a surgery. The extent of the surgery - from hemithyroidectomy to thyroidectomy. After the intervention, the thyroid tissue was removed for immunohistochemical studies no later than 30 minutes after the operation. In patients with TA we also took unchanged tissue of the lobe of the TG and adenomatous tissue for the study. In patients with AIT the tissue from the both lobes and from the isthmus was taken. Pieces of tissue weighing 100-300 mg were transported on ice to a laboratory and immediately cut into 4-6 pieces weighing an average 50-70 mg each. After the partition they were closed in a special plastic container and stored at -70 ° C until basic research was performed.

All patients' surgical material (tissue) was used to prepare cell suspension by painting thyrocytes with monoclonal antibodies (MAbs) to membrane receptors and intracellular proteins. We used ICA to estimate the parameters of apoptosis and proliferation produced by the firm «CALTAG» (Austria) - Bcl-2-Fits, Fas-PE-Cy5, FasL-PE-Cy5 and p53-Fits and Ki-67-PE of the firm «DAKO» (USA).

The density of expression of the membrane (intracellular) receptors (proteins) was evaluated in standard units (St. Un.) according to the average intensity of fluorescence light (MFI), which was proportional to the channel number, measured in logarithmic mode.

When counting cells we evaluated proliferation and apoptosis indices in research areas, using gating (Fig. 1), in which we determined the window, where the cells under 25 microns passed.

We determined the number of cells and their density with markers distributed on the surface of cells, Fas, FasL and intracellular proliferation marker Ki-67 and apoptosis bcl-2, p53. Phenotyping was performed on flow cytofluorimeter counting 100,000 events in the sample and calculating the relative number of cells and measuring the density of expression of receptors (proteins) in cells or groups of cells.

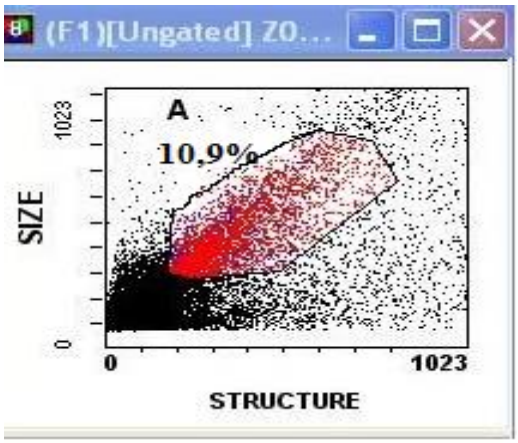

Fig. 1. Histogram of the area of research of heterogeneous suspension of the thyroid tissue with limited area of gating (A).

Digital data (histograms) as files (LMD) were analyzed by means of a special analytical program CXP ver.2.2 obtaining the results of the research.

We also studied small groups of cells formed with possible combinations: p53 / Ki-67, p53 / Fas, bcl-2 / Ki-67, bcl-2 / Fas, Fas / Ki-67, p53 / FasL, Fas / FasL, Bcl- 2 / FasL.

Results and discussion

Patients with AIT and TA did not differ by age (34,2 ± 10,33 and 38,0 ± 10,62 years respectively , p = 0.12), body mass index (BMI 23,5 ± 2, 71 and 24 3 ± 4,88 kg / m2, p = 0.43, respectively), free T3 rates (4,4 ± 0,91 and 4,4 ± 0,93 ng / L, p = 0.93), while content of free T4 in plasma was lower in patients with AIT than those with TA (16,6 ± 2,02 and 12,9 ± 3,42 mmol / L, p <0.0001), with higher TSH concentrations (1, 9 ± 0,76 and 4,9 ± 3, 51 mU / L, p <0.0001) and AT-TPO (11,9 ± 13,92 and 255,7 ± 340,58 mU / L, p = 0 , 0009), respectively. In general, the differences between the groups were natural and proved autoimmune destruction and the function downward trend against the background of AIT in patients of the first group.

Results of the study in figures are shown in Fig. 2.

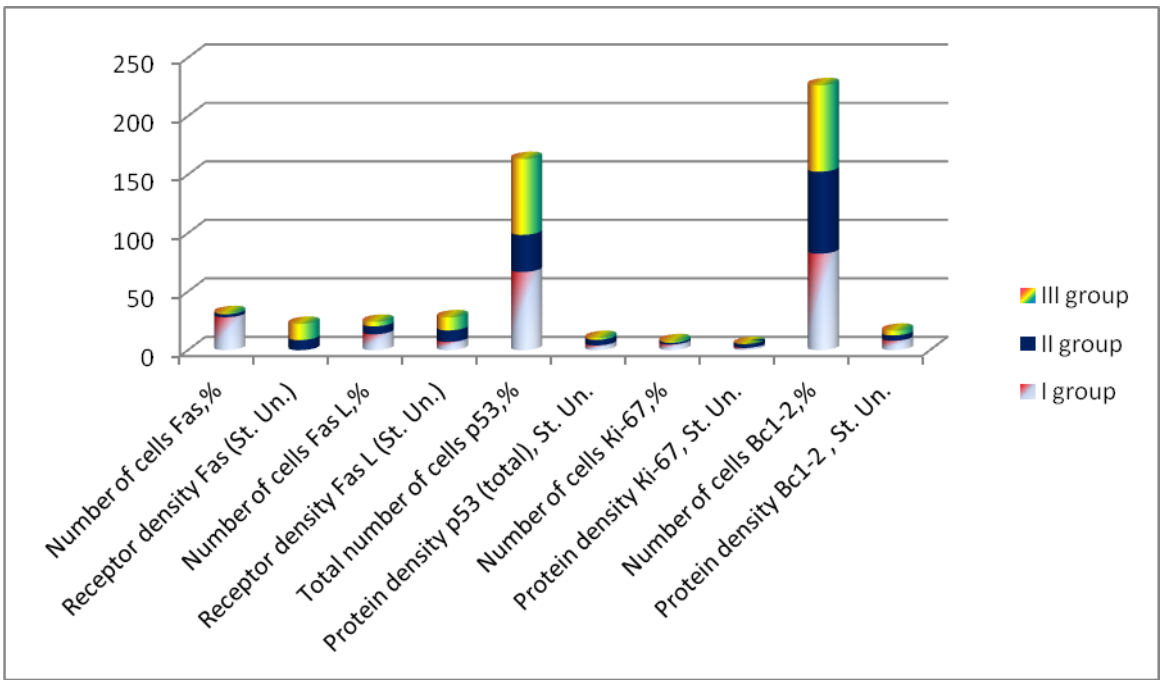

Fig. 2. Markers of apoptosis and proliferation in AIT, TA and morphologically unchanged areas of the thyroid tissue.

The patients of group I showed a high likelihood of increasing the number of cells with a marker Fas and Fas-L compared to those with TA and especially to the tissue of an unchanged lobe. It is indicative of a high probability of the development of Fas-induced apoptosis of the thyroid cells in AIT.

The study of intracellular proliferation marker Ki-67 in patients with thyroid diseases showed that the number of cells carrying this marker was higher by 68.2% in AIT than in TA ($p = 0.0001$) and by 76.3% than in unchanged thyroid tissue of the opposite thyroid lobe ($p = 0.0001$). Thus, we found a direct relationship between the severity of protein expression Ki-67 and Fas receptor in patients with AIT.

Total number of cells with p53 oncosupressor was higher in the group of patients with AIT than in patients with TA by 52.6% ($p = 0.001$). Instead p53 receptor density was greater in patients with TA (4,79 ± 1,09 St. Un.) than in those with AIT, where the rate was 4,07 ± 0,28 St. Un. At the same time, the density of receptors p53 in morphologically unchanged tissue was 2.5 times lower than in patients with AIT, and more than 3 times - than in patients with TA. According to the literature, the loss of function of p53 protein was found in about 50% of cases of human cancers, including thyroid cancer [13-17].

The data indicate the possibility of using p53 as a factor of differentiation between AIT and malignant thyroid diseases.

Total number of cells with the protein Bcl-2 was higher by 15.14% (p = 0.05) in patients with AIT than in patients with TA and by11.5% (p = 0.05) than in morphologically unchanged tissue and was accompanied by a high density of intracellular protein distribution by 45.5% (p = 0.001) and 51.85% (p = 0.001), respectively. The high level of this antiapoptotic value suggests containment of the processes initiating apoptosis and prolongs the survival term of thyrocytes.

Assessment of proliferation marker Ki-67 in thyroid tissue in patients with AIT showed the presence of more cells with intracellular protein Ki-67 than in the group of patients with TA, with a relatively low density distribution of the protein in Ki-67-positive cells. According to the literature, if we take the level of 20% for the line between benign and malignant thyroid tumors, then, using the Ki-67, we can determine the malignancy on the punctual material with a sensitivity of 82% and accuracy up to 84% [7-10].

In this regard, there is need for combined use of indicators of apoptosis and proliferation markers with the calculation of the threshold values and parameters of diagnostic efficiency for the differential diagnosis of benign and autoimmune thyroid disease (Fig.3).

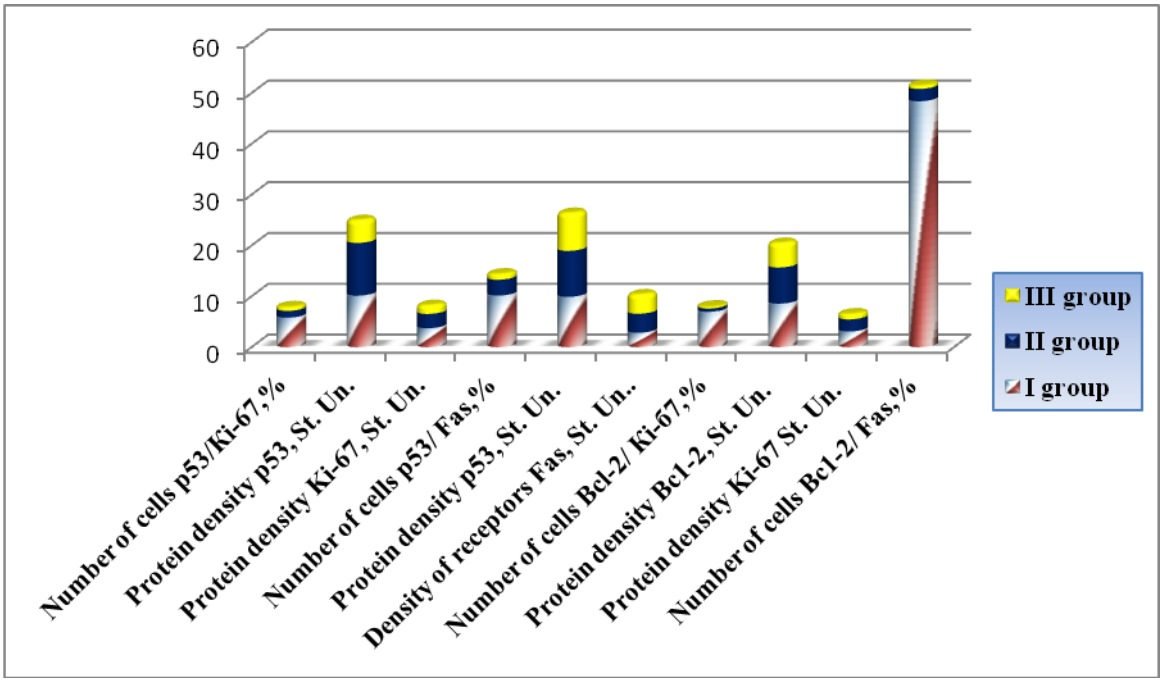

Fig. 3. Values in groups of cells and density of receptors in / on cells with combination of markers regulating apoptosis and proliferation in AIT, TA and morphologically unchanged tissue areas of the thyroid gland.

While studying combined values of apoptosis and proliferation in patients with AIT, we found more of all groups combined cells (p53 / Ki-67, p53 / Fas, bcl-2 / Ki-67, bcl-2 / Fas, Fas / Ki-67, p53 / FasL, Fas / FasL, bcl-2 / FasL) by 3-25 times compared with the group of patients with TA. The most essential differences were found in the group of cells bcl-2 / Fas, which had more

than 25-fold excess in patients with AIT compared to the patients with TA that can serve as an additional diagnostic and prognostic test for other basic instrumental, laboratory and morphological methods to improve diagnosis of AIT.

Conclusions.

1. Patients with AIT experience Fas-induced apoptosis of the thyroid cells with pronounced expression of Fas on thyrocytes in the areas of lymphoid infiltration and thyrocytes destruction as well as an increase of the number of immune-reactive cells, expressing Ki-67 which is indicative of the compensatory reaction as to the regeneration of the thyroid saved follicular epithelium.

2. Pronounced expression of Bcl-2 in the thyroid lymphocytes in patients with AIT prevents from involving the cells into apoptosis process and prolongs their survival time, which, undoubtedly, plays an important role in the morphogenesis of tumors diseases of the lymphoid tissue and a long course of the processes of the thyroid epithelium death and regeneration might contribute to carcinogenesis.

3. Finding the values (number of cells, density of receptors/proteins expression) of the main and combined markers, apoptosis regulators and proliferative ability of the experimental cellular suspension (p53 / Ki-67, p53 / Fas, bcl-2 / Ki-67, bcl-2 / Fas, Fas / Ki-67, p53 / FasL, Fas / FasL, bcl-2 / FasL) serve as additional diagnostic and prognostic tests for the main instrumental, laboratory and morphological methods of study, which improves their diagnostic accuracy.

4. Prospects for further research

The study of processes of apoptosis and proliferative activity in aspiration tissue sampling of the thyroid gland for the differential diagnostics of the nature of the pathology in the TG is perspective.

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