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## NEW PROGNOSTIC MARKERS OF NODULAR FORMS OF GOITER COMBINED WITH AUTOIMMUNE THYROIDITIS

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### Abstract

**Background:** Based on the results of a histological study of removed TG tissue NGAIT was found in 10,4% of the patients [1, 2]. At the same time, we observe processes of both thyroid epithelium metaplasia and lymphoid tissue hyperplasia that, undoubtedly, can be considered as an optional precancerous condition [3-8]. The option when cytomorphological and immunocytochemical examinations are carried out sequentially, using the same smear of a puncture material is optimal for PCE [9].

**Methods:** We have carried out an immunohistochemical study by means of monoclonal antibodies against Fas, FasL, Bcl-2, P53 and Ki67 antigens using the thyroid gland puncture material.

**Results:** The results showed the degree of proliferative activity in the thyroid tissue in NGAIT. We found a high proliferative activity of lymphoid tissue, moderate proliferative activity of thyroid epithelial cells in the area of lymphoid infiltration and a low one – beyond the latter.

**Conclusions:** The pronounced expression of Fas and FasL on the thyroid epithelial

cells in the area of lymphoid infiltration indirectly indicates that NGAIT causes the processes of thyroid epithelial cells apoptosis due to the immunity. Increasing the number of immunoreactive cells expressing Ki67 in the area of lymphoid infiltration and destruction of thyroid epithelial cells, are indicative of follicular epithelial regeneration as a compensatory-adaptive response of the organ. A pronounced Bcl-2 expression in lymphocytes prevents the cells from entering apoptosis and prolongs the cell survival time. There was a high expression of p53 protein in the nuclei of thyroid epithelial cells and follicular lamina, which can be explained by mutations in the gene p53, which allows the cells to find tolerance to apoptotic action of the immune system effectors.

**Abbreviations:** NGAIT - nodular goiter combined with autoimmune thyroiditis, TG – thyroid gland, PCE – preoperative cytological examination.

**Key words:** nodular goiter combined with autoimmune thyroiditis, fine needle biopsy, apoptosis, proliferation, thyroid gland.

### **Introduction**

In modern literature there are many publications dealing with a study of nodular goiter combined with autoimmune thyroiditis (NGAIT) morphology [1 - 5]. However, some issues remain unresolved including that about the role of autoimmune thyroiditis (AIT) in the development of tumor processes. According to the literature, AIT leads to metaplasia processes in the thyroid epithelium, hyperplasia of lymphoid tissue, which undoubtedly can be considered as an optional precancerous condition [4 - 8].

The information that papillary cancer and lymphomas occur three times more frequently in patients with NGAIT confirms this idea [3, 5 - 8].

The total accuracy of clinical, instrumental and laboratory diagnostic methods for the establishment of morphological origin of nodular new growths in the TG even in the most daring conclusions does not exceed 80% [1 - 8]. This result cannot be satisfactory either for surgeons (unjustified over diagnosis of thyroid cancer) or for endocrinologists (inadequate and ill-timed selection of patients for surgical treatment) [10, 11].

Unfortunately, the chemical reagents used in the preparation of drugs for morphological studies by a standard method, block most of the antigenic determinants. That is why immunocytochemical and morphological studies of the biopsy material is performed on individual drugs, which leads to additional needle biopsies and prevents from the morphological identification of the cells reacting with antibodies. Instead, the best for PCE is the option when cytomorphological and immunocytochemical study is carried out consistently

on the same smear of a puncture material [12, 13].

One of the mechanisms of malignant transformation and progression is a cell cycle dysregulation with apoptosis inhibition and proliferation activation [14 - 20].

Membrane receptors Fas and Fas-L as well as Bcl-2 proteins are usually attributed to apoptosis regulation markers; and some researchers give the key role to Bcl-2 in the regulation of apoptosis [14 - 15]. Besides the proteins of bcl-2 class the suppressor gene p53 a nuclear phosphoprotein is also an apoptosis regulator [14, 17, 18]. This gene may occur in two types: p53 "wild" type, is believed to stimulate apoptosis, whereas mutant p53 has a similar effect on apoptosis, like Bcl-2 protein, which inhibits a programmed cell death. There are about 500 mutations of this gene [18]. Mutations of this protein in patients with malignant, benign and autoimmune thyroid diseases have not been studied sufficiently [11, 18].

In addition to the factors of apoptosis, proliferation activity provides a very important information to determine the nature of precancerous condition and its prognosis in terms of malignancy. One of immunohistochemical markers of proliferation is the antigen Ki-67. It was shown that Ki-67 is on the cellular nuclei at all stages of the life cycle except G0 and G1 early stages. After a cell leaves the mitotic cycle the antigen is not detected [14 - 17, 20]. The expression of these molecules can be detected by standard immunocytochemical methods that are simple enough to integrate into the process of fine needle aspiration biopsy (FNAB). Using additional diagnostic techniques such as immunohistochemistry, can assist in the interpretation of "uncertain" changes, but currently there are not any universally adopted standard molecular markers [10, 11, 13, 15].

It is quite necessary to solve these problems, because the correct choice of treatment strategy, timely surgical treatment and therefore the patient's survival depend largely on the accuracy of PCE [11, 12].

That is why our aim was to study the processes of proliferation and apoptosis in thyroid puncture material under NGAIT using immunohistochemical method of investigation as well as determining the proliferative activity index.

### **Material and methods**

We examined 75 women with nodular NGAIT during 2015-2016. The diagnosis was made clinically, by laboratory findings (thyroid peroxidase antibodies TPOA) - 60-250 U / ml thyroglobulin antibody (TGA) - 60-500 U / ml, thyroid-stimulating hormone (TSH) - 4.10 mU / L) by ultrasound and it was confirmed histologically after the surgery.

The study did not include patients with hyperthyroidism, manifest hypothyroidism,

arterial hypertension and cardiovascular disease, severe somatic pathology or after the menopause.

All the patients were performed FNAB of the TG nodes controlled by ultrasound, while making at least three punctures of one node based on the fact that the percentage of inadequate punctates decreases depending on the number of punctures in the proportion when 1 puncture is 16%, 2 punctures are 5 3%, 3 punctures are 4%, 4 punctures are 2.6% [10].

While preparing the smears we used a method of restoration of antigen determinants activity developed and patented in the laboratory of the V. I. Komisarenko endocrinology Institute. It enables to combine cytomorphological and immunocytochemical researches in one cytological preparation and provides a possibility to compare morphological and immunocytochemical characteristics of certain cellular elements [9].

This method gives good results on the drugs that were kept after staining no more than three days. After this period, the results were unstable, which is due to the oxidization processes in some chemical compounds in the air [9,12,13]. To start an immunohistochemical reaction we used monoclonal antibodies to the following antigens: Mouse Human Ki-67 FITC Clone MIB-1; Anti-p53 Protein Monoclonal Antibody, FITC Conjugated, Clone DO-7; Mouse Anti-Human Apoptosis Regulator Bcl-2 (BCL2) Monoclonal, Unconjugated, Clone 124 antibody; Mouse Anti-Human CD95 Monoclonal Antibody, Unconjugated, Clone FAS18; Mouse Anti-Human CD95L Monoclonal Antibody, Unconjugated, Clone NOK-1 by Dako Denmark A/S.(Denmark)

The results of immunohistochemical reaction were evaluated by means of semiquantitative analysis, proposed by A.K. Khmelnytsky, according to the intensity of color "+ -" - small "+" - poor, "++" - moderate, "+++" - pronounced [13]. Assessment of immunoreactive cells was calculated by the formula  $(Fas, FasL, Bcl-2, P53) = N1 / N2 \times 100\%$ , where N1 is the number of immuno-positive cells to Fas, FasL, Bcl-2, P53 receptors, N2 - the total number of the cellular nuclei per 1 square millimeter. Assessment of the IPA was calculated by the formula  $= NKi67 / N \text{ total} \times 100\%$  where NKi67 is the total number of nuclei immune-positive to the protein Ki67, N total is the total number of nuclei per 1 mm<sup>2</sup>. Morphometric analysis was performed by means of the microscope Bresser BioScience Bino (Germany) with a digital camera Nikon DS-Fil, personal computer with installed software NIS-Elements F 3.2.

### **Results and discussion**

The immunohistochemical study found that in all 75 cases (100%) the expression of Ki67 in thyroid epithelial cells was weak "+" and moderate "++" by nature, which manifested

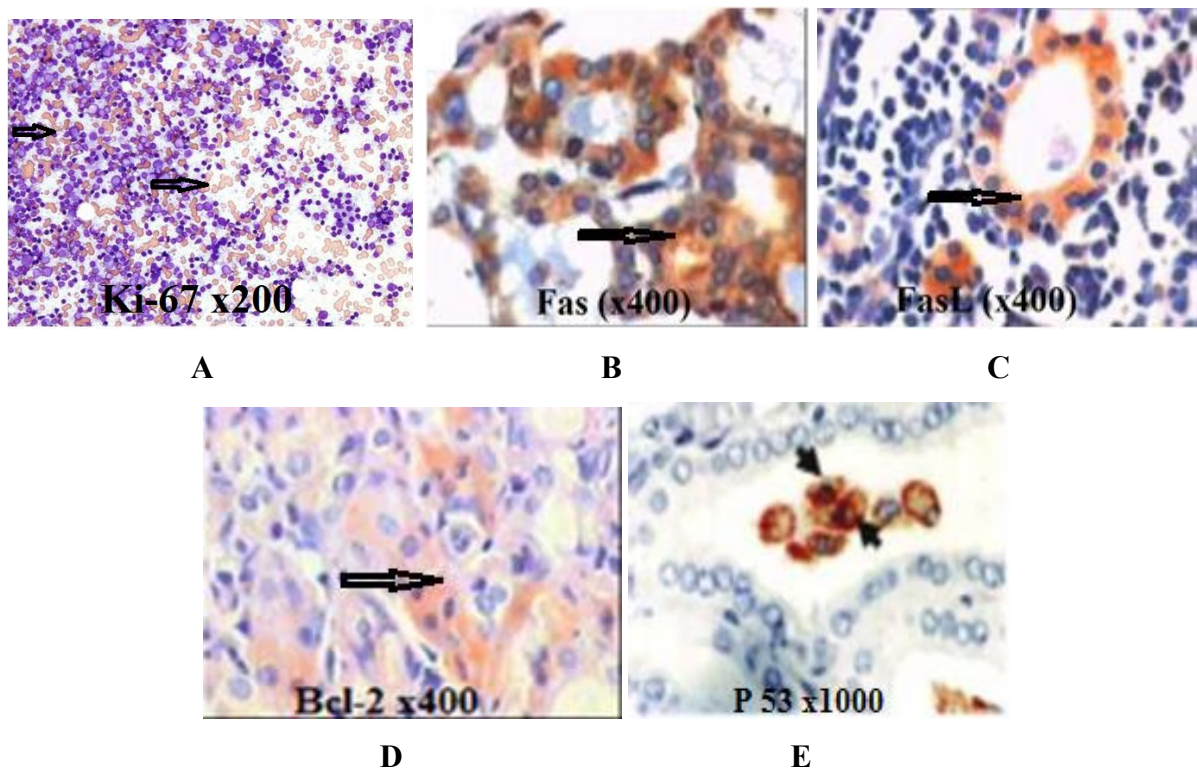
itself in the form of brown or light brown staining of nuclei in the thyroid epithelial cells. The number of immunoreactive cells in the preparation was distributed unevenly. In the thyroid epithelial cells, near the areas of thyroid epithelium destruction, near lymphoid infiltration there was an increased expression of Ki67, IPA varied from 9.75 to 11.37% (median was 10.56%). In calculating the number of immunoreactive thyroid epithelial cells which were beyond the lymphoid infiltration, proliferation index reduced from 2.5 to 3.7% (median 3.15%). The lymphoid tissue was characterized by a brown color and a high percentage of immunoreactive cells - from 93 to 95% (median 94%) (Fig. 1, A).

We have also found a significant number of thyroid epithelial cells expressing Fas. The cytoplasmic membrane of thyroid epithelial cells was characterized by a brown «++» and deep brown «+++» coloring. The number of immunoreactive cells ranged from 47 to 63% (median 55%). It should be noted that a significant expression "+++" was observed in the areas with a pronounced lymphoid infiltration. FasL expression was higher "+++" in follicles and adjacent areas of lymphoid infiltration "++" which averaged 46.10% in the cell ratio (Fig. 1, B, C). Co-expression of Fas and FasL in the areas of lymphoid follicles infiltration around thyroid epithelial cells indicates that Fas and FasL are not directly involved in apoptosis of thyroid epithelial cells but induce this process through the development of proapoptotic cytokines.

Bcl-2 was slightly «-» expressed by the thyroid epithelium unlike the lymphoid tissue, whose cytoplasm became brown «+++». When the lymphoid follicles were formed without clear boundaries, the lymphoid tissue of the thyroid gland was evenly colored in the center and the periphery of follicles. When mature follicles were formed, there was no coloring in the center but the mantle zone of lymphoid follicles was intensely colored. (Fig. 1, D). The lymphoid tissue beyond the nodules was being intensively expressed by bcl-2 in the area of thyroid follicles and in the stroma of the gland. When analyzing the number of cells expressing bcl-2 we found from 87 to 93% of immunoreactive cells (median 90%).

There was a high expression of protein p53 in the nuclei of thyroid epithelial cells and in the follicular lamina, at the same time there was a prominent positive reaction with the nuclei colored deep brown. (fig.1, E). Staining with this marker was local by nature, and there were no more than 7-9 nuclei in one field of view. The concentration of immunoreactive cells was from 64 to 71% (median 65, 5 %). An increased density of p53-positive cells can be explained by mutations in the gene p53, which allows the cells to find tolerance to apoptotic action of the immune system effectors [14, 15, 18].

**Fig. 1**



### **Conclusions**

1. A pronounced expression of Fas and FasL on the thyroid epithelial cells, in the areas of lymphoid infiltration is indicative of the fact that in case of NGAIT immunologically caused processes of the thyroid epithelial cells apoptosis occur.
2. Co-expression of Fas and FasL in the areas of the lymphoid infiltration around the thyroid epithelial cells indicates that Fas and FasL are not directly involved in apoptosis of the thyroid epithelial cells, but they induce this process by producing proapoptotic cytokines.
3. Increasing the number of immunoreactive cells expressing Ki67 in the area of the lymphoid infiltration and destruction of the thyroid epithelial cells is indicative of the follicular epithelium regeneration as a compensatory-adaptive response of the organ.
4. A pronounced expression of bcl-2 in the lymphocytes prevents the cells from entering apoptosis as well as prolonging their survival time, which, undoubtedly, plays an important role in morphogenesis of the lymphoid tissue tumor diseases and prolonged processes of apoptosis and regeneration of the thyroid epithelium can also contribute to cancerogenesis.
5. An increased density of p53-positive cells can be explained by mutations of p53 gene, which allows the cells to find tolerance to an apoptotic action of the immune

system effectors.

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