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IMMUNOHISTOCHEMICAL CHARACTERISTICS OF THYROID GLAND CHANGES IN PATIENTS WITH AUTOIMMUNE THYROIDITIS

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The purpose of the study was to perform an immunohistochemical study of autoimmune changes in the thyroid gland in autoimmune thyroiditis patients who received various treatment methods. In the main group, immunohistochemical studies were performed on 30 patients. The control group consisted of six patients in whom the histochemical examination was performed postmortem. Statistically significant differences were found between the forms of autoimmune thyroiditis according to Ki–67(+) ($F=1428.687$, $p<0.050$). According to the specific amount of Ki–67(+), p53(+), and thyroglobulins, there is a statistical difference between forms of autoimmune thyroiditis in cells. A correct assessment of the proliferative activity of the thyroid gland tissue during immunohistochemical studies with the determination of biomarkers Ki–67(+), p53(+) and thyroglobulin may indicate the preservation of the apoptosis mechanism, which will allow to predict the risk of recurrence of the disease and the malignancy of nodular forms of autoimmune thyroiditis.

Key words: autoimmune thyroiditis, thyroglobulin, proliferative activity, biomarker, immunohistochemical studies.

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ІМУНОГІСТОХІМІЧНА ХАРАКТЕРИСТИКА ЗМІН ЩИТОВИДНОЇ ЗАЛОЗИ У ХВОРИХ НА АУТОІМУННИЙ ТИРЕОЇДИТ

Метою дослідження було імуногістохімічне дослідження аутоімунних змін щитовидної залози у хворих з діагнозом аутоімунний тиреоїдит, які отримували різні види лікування. В основній групі імуногістохімічні дослідження проведено у 30 хворих з діагнозом аутоімунний тиреоїдит. Контрольну групу становили шість пацієнтів, яким гістохімічне дослідження проводилося посмертно. Для перевірки статистичної значущості відмінностей використовували критерії ANOVA (F), Kruskal – Wallis – H, Wilcoxon – T, Mann – Whitney – U та Student – T. Статистично значущими вважалися відмінності при $p<0,050$. Виявлено статистично значущі відмінності між формами аутоімунного тиреоїдиту Ki-67(+) ($F=1428,687$, $p<0,050$). За питомою кількістю Ki-67(+), p53(+) та тиреоглобулінів спостерігається статистична різниця між різними формами аутоімунного тиреоїдиту у клітинах. Правильна оцінка проліферативної активності тканини щитовидної залози при імуногістохімічному дослідженні з визначенням біомаркерів Ki–67(+), p53(+) та тиреоглобуліну може свідчити про збереження механізму апоптозу, що дозволить прогнозувати ризик рецидиву захворювання та ризик малігнізації вузлових форм аутоімунного тиреоїдиту.

Ключові слова: аутоімунний тиреоїдит, тиреоглобулін, проліферативна активність, біомаркер, імуногістохімічні дослідження.

Autoimmune thyroiditis (lymphocytic thyroiditis) is an organ-specific disease characterized by chronic inflammation of the thyroid gland. A characteristic morphological sign of autoimmune thyroiditis is lymphoplasmacytic infiltration of the thyroid tissue of the gland, with obligatory autoimmune inflammation [3, 10]. Autoimmune thyroiditis (AIT) is the most common disease of the thyroid gland (TG), which occupies from 20 to 46 % of all thyroid pathology. The incidence ranges from 5 % to 10 % in the entire elderly population and from 0.1 % to 1.2 % in children. In the literature sources of the last decades, a trend of up to a tenfold increase in the incidence of autoimmune thyroiditis has been recorded [14].

Currently, none of the methods of preoperative research provide a high degree of reliability in the diagnosis of AIT, as a result of which the importance of histological examination increases. There is no generally accepted morphological classification [1, 7]. The question of the attitude to autoimmune thyroiditis remains open; the cause and development mechanisms are not sufficiently elucidated. There are no clear morphological criteria for diagnosing the disease in the literature. According to most authors, the main signs in the diagnosis of AIT are lymphoplasmacytic infiltration, B – cell metaplasia of the epithelium, and stromal fibrosis of the thyroid gland. The question remains whether B cells are found in normal thyroid tissue. The origin and functional significance of these cells in AIT is not clear. Morphological criteria for disease activity have not been elucidated [1, 13].

The results of immunohistochemical studies correspond to the pathogenesis of autoimmune thyroiditis; therefore, the occurrence of thyroiditis is associated with dysfunction of the immune system. Immunodeficiency is a consequence of impaired differentiation of T – lymphocytes, namely, a decrease in the suppressive and an increase in the helper function of these cells. Information is transmitted to B – lymphocytes and plasma cells that produce organ-specific antibodies: antibodies to thyroglobulin (Anti – TG) and thyroid peroxidase (Anti – TPO) [2, 9].

Patients with autoimmune thyroiditis have a higher risk of developing “thyroid cancer” than those who do not have the disease. At the core of such development of the pathological process are genetic mechanisms. Histological and immunohistochemical studies show that an increase in the expression of Ki–67(+) (a marker of cell proliferation), p53(+) (a marker of cell apoptosis), and thyroglobulins in autoimmune thyroiditis is a reflection of an increase in the proliferation processes of thyrocytes [4, 11]. Furthermore, as you know, proliferation is a direct path to cellular dedifferentiation and dysplasia of thyrocytes. The study of the proliferative activity of thyrocytes in autoimmune thyroiditis makes it possible to predict the course of the disease [12, 15]. It is important to emphasize that in the nodular form of AIT, the highest expression is observed in the reproduction centers of lymphoid follicles. In the diffuse form of AIT, a decrease in nucleolar organizers in one thyrocyte by 25 % was noted, which indicates a decrease in functional cellular activity. In the diffuse form of AIT, proliferative activity decreases in tissue areas with atrophic changes in follicles [5, 6].

Therefore, in different forms of AIT, the proliferative activity of thyrocytes is also different. Despite the study of the main pathogenetic mechanisms of autoimmune thyroiditis, morphological criteria, pathogenesis, and determination of immunohistochemical results of the disease have not been studied enough. Thus, the increase in the frequency of AIT, the difficulties of diagnosis, and the conflicting opinions of researchers in approaches to the treatment of this category of patients indicate the relevance of this problem.

The purpose of the study was to perform an immunohistochemical study of autoimmune changes in the thyroid gland in patients diagnosed with autoimmune thyroiditis who received various types of treatment methods.

Materials and methods. Research work is based on the study of examination and treatment results of patients at the clinical base of the Scientific Center of Surgery named after Academician M.A. Topchubashov for 2017–2021. With the help of immunohistochemical studies, pathomorphological changes occurring in the thyroid gland in autoimmune thyroiditis were studied. The research was carried out on patients with various forms of autoimmune thyroiditis who received various treatment methods. In the main group, immunohistochemical studies were performed on 30 patients diagnosed with autoimmune thyroiditis. The control group consisted of six (6) patients in whom the histochemical examination was performed postmortem. The sample (tissue) of the thyroid gland was taken from 6 deceased people (2 men and 4 women). The causes of death of these people were diseases not related to thyroid diseases.

Immunohistochemical studies were performed by the peroxidase-antiperoxidase method according to standard diagnostic protocols. For immunohistochemical verification of the process, the monoclonal antibody Dako Cytomation (chromogranin) was used. For visualization, a highly sensitive “ABC System” and EnVision TM Kit were used. The preparation of solutions and the reaction on the control and altered tissues were carried out in accordance with the recommendations of the manufacturer.

Biomarkers (Thermo Fisher Scientific) were also used: monoclonal rabbit antibodies against Ki–67 (Clone SP6, working dilution of primary antibodies 1:200), p53 (apoptosis protein) (Clone SP5, working dilution of primary antibodies 1:100), monoclonal mouse antibodies against human thyroglobulin (Clone SPM517, working dilution 1:200).

Accounting for the expression of biomarkers was carried out by a quantitative method. The method of unmasking antigens and the time of incubation of primary antibodies was carried out in accordance with the recommended protocol of the manufacturer. The polymer system Ultra Vision Quanto detection systems (Thermo Fisher Scientific) was used as a detection system. For the reliability of the results obtained, positive and negative antibody controls were used. A 1 % solution of 3,3 diaminobenzidine tetrachloride was used as a chromagen.

During the cytological study, we studied the quantitative composition of cells using the morphometry method. In cytological preparations, an analysis of the quantitative composition of neutrophils, macrophages, lymphocytes, fibroblasts, and other cells was carried out. To assess the effectiveness of treatment, in addition to immunohistochemical studies of the studied material, a fine-needle aspiration puncture biopsy (FNAB) was performed with cytological examination using the Bethesda system.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Scientific Center of Surgery named after Academician M. A. Topchubashov, Republic of Azerbaijan, Baku (Minutes No. 2, dated May 16, 2019).

All calculations were carried out on the EXCEL – 2019 spreadsheet. Statistical analysis was performed using the IBM SPSS Statistic software package version 26. Depending on the nature of the

indicators, ANOVA (F), Kruskal – Wallis – H, Wilcoxon – T, Mann – Whitney – U, and Student – T criteria were used. Differences were considered statistically significant at $p<0.050$.

Results of the study and their discussion. During a general examination of the preparations, the tissue of the thyroid gland of patients with AIT has a characteristic appearance: against a dark background of dense lymphoplasmacytic infiltration, small follicles, strands, and individual cells of a large light oxyphilic epithelium (Ashkenazi – Gurtl cells) are clearly distinguished. The diffuse nodular form of AIT in most patients is characterized by signs of an autoimmune process in combination with a tendency of the thyroid gland to grow and enlarge with the formation of nodular and multi – nodular formations. The nodes have a mostly colloidal structure, atrophic and sclerotic changes in the parenchyma, lymphoplasmacytic infiltration and oxyphilic cell transformation of the follicular epithelium are detected around them. In the diffuse – pseudonodular form of AIT, the described changes persist in most patients, but they occur less frequently. The diffuse form of AIT is characterized by pronounced deformation and changes in the epithelium, which occupies small areas in the gland tissue. Small foci are lymphoid infiltrates and lymphoid follicles with reproduction centers. Focal lymphocytes are mainly represented by T – lymphocytes. Lymphocytic infiltrates are small.

The atrophic form of AIT is characterized by pronounced sclerotic changes and lymphoplasmacytic infiltration of the parenchyma of the gland. Changes in fibrous structures are permanent. At the same time, interlobular connective tissue layers grow in the stroma of the gland, and thus powerful fibrous cords are formed that separate the lobules of the gland into smaller sections.

For a more detailed analysis of some morphological features of pathological changes in the thyroid gland in patients with AIT, we studied the proliferative activity of thyrocytes using monoclonal antibodies of the Ki–67 protein (a marker of cell proliferation), p53 protein (a marker of thyrocyte apoptosis) and thyroglobulin. In immunohistochemical studies, B – cells gave a bright expression for chromogranin in all cases. Accounting for the expression of chromogranin A was carried out by a semi – quantitative method. The study of the results of immunohistochemical examination of the thyroid gland was carried out in patients with various forms of autoimmune thyroiditis (Table 1).

Table 1

The results of immunohistochemical studies in various forms of autoimmune thyroiditis (in absolute and %)

Form	Specific quantity of Ki–67(+) cells %	Specific quantity of p53(+) cells %	Specific quantity of thyroglobulin (+) cells %
Diffuse n=6	13.85±0.40	16.0±0.28	24.31±0.32
Diffuse –pseudonodular n=6	17.37±0.31	21.01±0.23	58.33±0.35
Diffuse – nodular n=6	21.44±0.38*	27.42±0.48	67.28±0.68**
Atrophic n=6	12.00±0.36	14.71±0.51	19.32±0.59
Thyrotoxic n=6	16.46±0.57	31.25±0.59**	48.25±0.59
Control n=6	5.39±0.48	4.58±0.53	16.84±0,50

Note: the values of the quantitative characteristic in the indicated group are statistically significant. *($p<0.050$), **($p\leq0.001$)

According to the ANOVA (F) criteria, statistical differences were revealed between the forms of autoimmune thyroiditis (control, thyrotoxic, atrophic, diffuse – nodular, diffuse – pseudonodular) according to Ki–67(+) ($F=1428.687$, $p<0.050$).

The maximum value is observed in the "diffuse – nodular" group (mean value =21.44), and the minimum value is observed in the "control" group (mean value=5.392). According to the Kruskal – Wallis – N criterion, a statistical difference is revealed between the forms of autoimmune thyroiditis (control, thyrotoxic, atrophic, diffuse – nodular, diffuse – pseudonodular) for p53 (+) and thyroglobulins (+) ($H=19.323$, $p<0.001$**). The maximum value is observed in the "thyrotoxic" and "diffuse-nodular" groups (mean value 31.25; 67.28, respectively), and the minimum value is observed in the "control" group (mean value 4.58; 16.84, respectively).

When statistically evaluating the results of immunohistochemical studies of the thyroid gland in various forms of autoimmune thyroiditis according to the criteria of Wilcoxon – T, Mann – Whitney – U, there is a statistical difference between different forms of autoimmune thyroiditis in the specific amount of Ki–67 (+), p53 (+), and thyroglobulin in cells (respectively, as $T_{emp.}$, $U_{emp.}=21$, $p=0.028$*).

High expression of Ki–67 protein was observed in the zone of lymphoid infiltration, in the reproduction centers of lymphoid follicles, and reached 21.44±0.38 %. In the interfollicular zone, expression decreased and amounted to 17.37±0.31 %. Statistical significance of differences with control material is indicated $p<0.050$. In the nodular form of autoimmune thyroiditis in the goiter-altered tissue of the gland, the expression of p53 is moderate (21.01±0.23 %), and in the foci of the proliferation of the follicular epithelium, the expression of p53 is high.

The indicators of cytoplasmic expression of thyroglobulin are reliable. In diffuse and atrophic forms of autoimmune thyroiditis, thyroglobulin expression is low (Fig. 1), respectively 24.31±0.32 % and 19.32±0.59 %. Compared with them, the thyroglobulin expression is high in the diffuse – pseudonodular form (58.33±0.35 %) and even higher in the nodular form in the changed areas of the goiter (67.28±0.68 %).

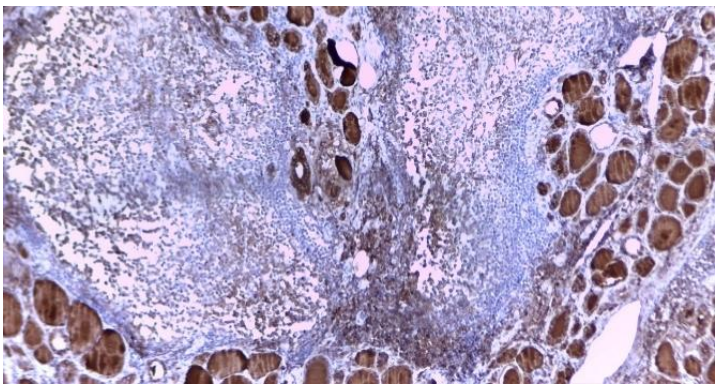

Fig. 1. Low expression of thyroglobulin biomarker in diffuse form of autoimmune thyroiditis. Patient N., a. 54. PAP method using antibodies to thyroglobulin. x200.

Thus, the study of proliferative activity of thyrocytes using monoclonal antibodies showed high expression of Ki–67(+) (marker of cell proliferation), p53(+) (marker of cell apoptosis), and thyroglobulins in patients with diffuse – nodular and diffuse – pseudonodular forms of autoimmune thyroiditis. Higher expression of biomarkers was observed, especially in the zone of lymphoid infiltration and in the reproduction centers of lymphoid follicles. In the diffuse and atrophic forms of autoimmune thyroiditis, a decrease in the expression of all three biomarkers is observed, which is related to the atrophy of the parenchyma of the thyroid gland and sclerotic changes in its stroma.

Currently, the literature sources contain a number of successful research papers on the study of thyroid diseases based on the study of immunogenetic and immunological factors. However, a number of unresolved problems associated with autoimmune thyroiditis remain. The etiological and pathogenetic aspects of the disease have not been studied enough; there is no generally accepted unified classification of the disease; modern reliable methods for diagnosing the disease, including immunohistochemical methods, remain insufficiently studied [1, 12].

The clinical picture of the disease is determined by the severity and prevalence of pathomorphological changes in the thyroid gland. In most cases, cytological examination of the punctate nodular formation of the thyroid gland allows you to determine the nature of the node. However, it is not always possible to verify the diagnosis of AIT by cytological signs. Sometimes thyrocytes of benign follicular neoplasms, according to morphological characteristics, do not allow reliable differentiation of a benign tumor – follicular adenoma – from follicular carcinoma cells [7]. The same facts significantly complicate the identification of pathological changes in cells in autoimmune thyroiditis. Sometimes the term “follicular tumor” combines all nodular formations in the thyroid gland, including those formed against the background of AIT. In practice, we can talk about a benign tumor – follicular or microfollicular adenoma, often combined with autoimmune thyroiditis; therefore, increasing the sensitivity and specificity of methods for diagnosing AIT is important from the point of view of further treatment tactics for this category of patients [1, 2].

The results of our study are consistent with the work of the authors, who managed to show that the principle of treatment of autoimmune diseases involves the suppression of the activity of the immune system and the reduction of lymphoplasmacytic infiltration of the thyroid tissue. Functionally active mononuclear cells that infiltrate thyroid tissue also actively accumulate photosensitizers, as well as tumor cells and microflora; therefore, it is possible to induce their apoptosis with the formation of apoptotic bodies, which macrophages recognize and phagocytose without damage to neighboring healthy cells, with the release of regulatory cytokines, which, together with the anti-inflammatory effect, allows testing photodynamic therapy in the treatment of various forms of autoimmune thyroiditis [8].

The study of the proliferative activity of thyrocytes using monoclonal antibodies made it possible to understand that in patients with diffuse – nodular and diffuse – pseudonodular forms of AIT, a high expression of biomarkers Ki–67, p53, and thyroglobulin is observed. The biomarker expression rates are especially high in the zones of lymphoid infiltration and the reproduction centers of lymphoid follicles. In atrophic and diffuse forms of autoimmune thyroiditis, the expressions of all three biomarkers are reduced, which is due to atrophy of the thyroid parenchyma and sclerotic changes in the stroma. When comparing histological and immunohistochemical parameters, a tendency for a decrease in the expression of markers in areas of atrophy of the parenchyma of the gland and a high expression in the foci of lymphoid infiltration, especially in the follicle reproduction centers is determined. The revealed pattern indicates a gradual progressive decrease in the proliferative activity of thyrocytes.

The strength of the study is the immunohistochemical study of autoimmune changes in the thyroid gland in patients diagnosed with autoimmune thyroiditis who received various types of treatment methods. For the final verification of the histological diagnosis and in order to avoid diagnostic errors, it is advisable to use immunohistochemical methods. The indicators of the expression of biomarkers Ki–67 (marker of cell proliferation), p53 (marker of cell apoptosis), and thyroglobulin are reliable. Given the results of other studies, it should be noted that the findings of the study are interesting and need further study on a representative sample on a larger scale.

Conclusions

1. There is a statistical difference between the forms of autoimmune thyroiditis (control, thyrotoxic, atrophic, diffuse – nodular, diffuse – pseudonodular) according to Ki–67(+) (F=1428.687, p<0.050). According to the specific amount of Ki-67(+), p53(+), and thyroglobulins, there is a statistical difference between different forms of autoimmune thyroiditis in cells ($T_{emp.}$, $U_{emp.}$=21, p=0.028*, respectively).

2. A correct assessment of the proliferative activity of the thyroid gland tissue during immunohistochemical studies with the determination of biomarkers Ki–67(+), p53(+) and thyroglobulin may indicate the preservation of the apoptosis mechanism, which will allow to predict the risk of recurrence of the disease and the malignancy of nodular forms of autoimmune thyroiditis.

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