Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
https://doi.org/10.33263/BRIAC102.201208
Original Research Article
Open Access Journal
Received: 09.10.2019 / Revised: 20.01.2020 / Accepted: 25.01.2020 / Published on-line: 03.02.2020

The activity of proliferation and apoptosis of thyrocytes in the thyroid tissue of patients of nodular goiter with autoimmune thyroiditis considering the polymorphism of the *BCL-2* (*RS*17759659), *CTLA-4* (*RS*231775), *APO-1/FAS* (*RS*2234767) genes

Michael I. Sheremet [1,*], Larysa P. Sydorchuk [2], Viktor O. Shidlovskyi [3], Volodimir I. Desiateryk [4], Anatoliy E. Kovalenko [5], Stanislav I. Shevchenko [6], Serhiy M. Zavgorodnyi [7], Nina P. Tkachuk [1], Antonina A. Piddubna [8]

[1]Surgery Department №1, Bukovinian State Medical University, Ukraina
[2] Family Medicine Department, Bukovinian State Medical University, Ukraina
[3]Surgery Department, I.Y. Horbachevsky State Medical University, Ukraina
[4] Department of Surgery, Traumatology and Orthopedics Faculty of Postgraduate Education of Dnipropetrovsk State Medical Academy, Ukraina
[5] Endocrine Surgery Department, Institute of Endocrinology and Metabolism V.P. Komisarenko of NAMS of Ukraina
[6] Department of General Surgery №1 Harkiv National Medical University, Ukraina
[7] Department of Surgery and Anesthesiology, Zaporizhzhya Medical Academy of Postgraduate Education, Ukraina
[8] Department of Clinical Immunology, Allergology and Endocrinology, Bukovinian State Medical University, Chernivtsi, Ukraine
*corresponding author e-mail address: ***Mihayl71@gmail.com*** | Scopus ID 57193774935

ABSTRACT

Nodular goiter with autoimmune thyroiditis is one of the most important problems of modern endocrinology, with inadequately studied etiological and pathogenic mechanisms of development. It is characterized by the lack of objective and reliable diagnostic methods, effective treatment methods, uncertain therapy or indications for the choice of treatment methods. A total we have examined 125 patients who were operated for a nodular endemic goiter with autoimmune thyroiditis. Investigated the activity of proliferation and apoptosis of thyrocytes in the thyroid tissue of patients of nodular goiter with autoimmune thyroiditis considering the polymorphism of the *bcl-2* (*rs*17759659), *ctla-4* (*rs*231775), *apo-1/fas* (*rs*2234767) genes. The expression/density markers - Fas/ FasL, Bcl-2, p53 and Ki-67 on the thyrocytes in the lymphoid infiltration and destruction areas, as well as in normal thyroid tissue (as a control) were studied. The number of immunoreactive cells, which expressed the above-mentioned regulating apoptosis and proliferation markers in NGAIT patients, depending on the genes polymorphism BCL-2 (rs17759659), CTLA-4 (rs231775) and APO-1/Fas (rs2234767) were counted. It was found that in NGAIT patients a few links of programmable thyroid cell killing of Fas-induced apoptosis were activated, and associated with the polymorphic cite of BCL-2 (rs17759659) gene and almost 6 times weaker with CTLA-4 (rs231775) gene, through enhanced expression of Fas and Fas L on the cells surface in lymphoid infiltration and destruction areas (stronger in GG genotype carriers of BCL-2 gene).

Keywords: *nodular endemic goiter; autoimmune thyroiditis; apoptosis; proliferation; genes polymorphism.*

Abbreviations: *NGAIT – nodular goiter with autoimmune thyroiditis; TG – thyroid gland; TPOAB – thyroperoxidase antibodies, TGAB – thyroglobulin antibodies.*

1. INTRODUCTION

One of the mechanisms of malignant transformation and progression is cell cycle dysregulation with apoptosis inhibition and activation of proliferation. Protein Ki-67, whose antibodies recognize DNA-related nuclear protein present in the nuclei of cells in 01-, 8-, 02- and M-phases and absent in the 0_0-phase, is a prospective intracellular marker of proliferation [1-6]. Protein p53 is a diagnostically significant tumor marker being a product the gene suppressor of a tumor p53 is expressed in all cells, activated by a damage to the genetic apparatus, as well as by the stimuli that could lead to such damage, or that serve as a signal of an unfavorable condition of cells (stress). Its activation results in the cell cycle arrest, DNA replication, and excessive stress signal in apoptosis [7-11]. The function of anti-apoptotic protein p53 is to remove the cells that are potentially oncogenic out of the pool. In almost 50% of cases of human cancers, the loss of protein p53 function is diagnosed [10-18].

Cell death is mediated through the interaction of the surface cellular receptors Fas / Fas ligand, or CD95L (type II of the transmembrane protein belonging to the family of tumor necrosis factor alpha (TNF), which is expressed on cytotoxic T lymphocytes) and gets activated through the apoptosis caspase trigger mechanism [19-20]. This is an important link to the pathological process to maintain the homeostasis of immune cells and immune defenses of the body. Using the Fas / FasL system, apoptosis is also an important means to destroy the cytotoxic T cells. [20-23].

Assessing the markers which regulate apoptosis (protein p53, *Bcl*-2, *Fas*-system) and proliferation (protein *Ki*-67), as well as their relationship with polymorphism of genes associated with apoptosis, the role of autoimmune reactions in this process has not been studied well enough and requires further research.

Therefore, the purpose of this phase of our work is the analysis of apoptosis and proliferation indices (expression/density

of markers Fas / FasL, Bcl-2, p53 and Ki-67 on thyrocytes in the areas of lymphoid infiltration and destruction of thyrocytes as well as in morphologically unaltered areas of the thyroid tissue (as a control), and counting the number of immunoreactive cells that express the above mentioned markers, which regulate apoptosis and proliferation for AIT and TA, using immunohistochemical method considering polymorphism of *BCL-2* (*rs*17759659), *CTLA-4* (*rs*231775) and *APO-1/Fas* (*rs*2234767) genes.

2. MATERIALS AND METHODS

During 2014-2019 we have examined 125 women complaining about discomfort in the neck. We evaluated the hormonal status (TSH, free T4 and free T3) ratio of antibodies to thyroglobulin (ABTG) 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. The 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 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 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.

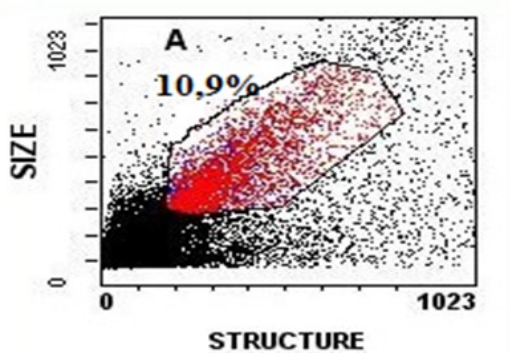

Figure 1. Histogram of the thyroid tissue heterogeneous suspension with limited gating area (A).

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.

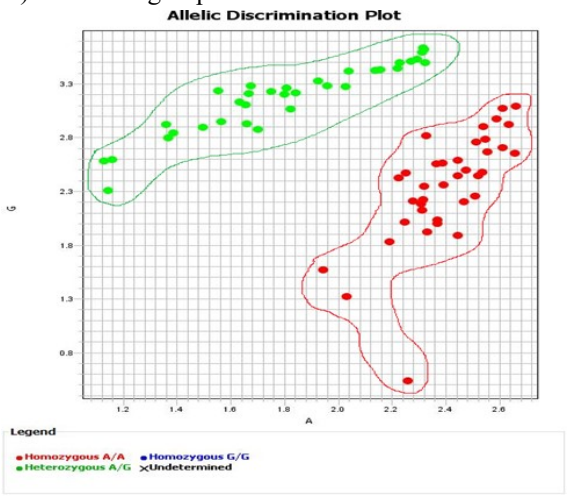

Figure 2. *CTLA*4 gene polymorphism (rs 231775) alleles discrimination.

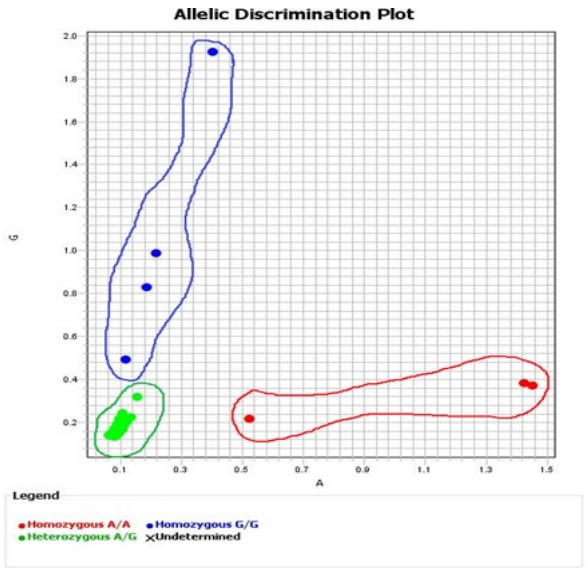

Figure 3. *BCL*-2 gene polymorphism (17759659) alleles discrimination.

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.

The processes of proliferation and apoptosis in thyroid tissue were studied in patients with NGAIT and compared with

norm for the region. It was identified in the comparison group in the study of thyroid tissue taken from 36 residents of Chernivtsi region, who died during an accident and accidents. The material was received at the Chernivtsi Pathological and Anatomical Bureau in accordance with the agreement on joint research between the Higher Medical University of Ukraine "Bukovyna State Medical University" and the "Pathological and Anatomical Bureau".

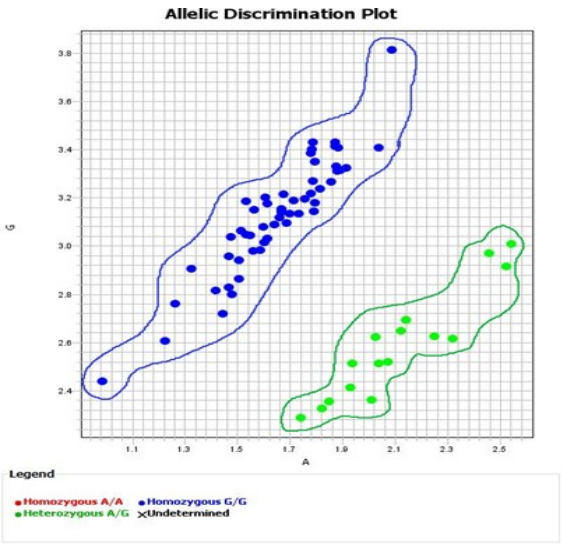

Figure 4. *FAS* gene polymorphism (rs 2234767) alleles discrimination.

Genetic studies performed in the laboratory of genetics at the State University of Medicine and Pharmacy "Nicolae Testemițanu", Chisinau (Republic of Moldova). DNA was extracted from whole venous blood lymphocytes. Venous blood was stored in test tubes, stabilized with K2-EDTA. Isolation and purification of DNA from the material obtained were performed according to methodological guidance of Thermo Scientific GeneJET Genomic DNA Purification kit (#K0721, Thermo Fisher Scientific).

Quantitative Real-Time PCR (RT-PCR).

Polymerase chain reaction (PCR) was performed in real-time (RT-PCR) using Taq-DNA polymerase and specific primers on QuantStudio 6 equipment, Applied Biosystems (USA), which allowed us to obtain amplicons to determine their number in "real time" and reduce the likelihood of diagnostic error. Analysis of the data was performed using the Quant Studio Real Time Software (Fig. 2-4).

Statistical Analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc, USA) software. Nominal data presented in the form of quantitative and percentages. For the genotypes distribution comparison used Pearson's criterion (χ^2). Analysis of qualitative data (categorical variables), risk of thyroid pathology development was assessed using a binary logistic regression model using the relative risk (RelR), risk ratio (RR) and odds ratio (OR) with 95% confidence interval [95% CI], chi-square test (χ^2) (df=1). The difference was considered reliable at $p<0.05$.

3. RESULTS

Cell number and density of the receptors with markers, distributed on the surface of Fas, FasL cells and intracellular proliferation markers Ki-67 and apoptosis of Bcl-2, p53 considering the polymorphic variants of *Bcl-2* (rs17759659) gene are shown in tables 2. The number of immunoreactive cells expressing transmembrane protein *Fas* on the surface is reliably higher in the homozygous carriers of minor G-allele of the *BCL-2* gene than in the owners of the main A-allele (AA- and AG-genotypes) by 18.54% ($p_{AA}=0.043$) and 36.18% ($p_{AG}=0.018$). As for other indices (the number of cells with receptors to *Fas L* and intracellular markers of apoptosis - p53, *Bcl-2* and proliferation – Ki-67 and the density of these markers both on the surface and inside the cells) considering the polymorphism of the *BCL-2* (rs17759659) gene, they were not determined. While comparing with the reference values of the control group, in general, we established in the patients with thyroid pathology a reliably higher number of cells with receptors to *Fas, Fas L, Bcl*-2 and Ki-67 ($p\leq0.055$-0.001). On the other hand, the density of receptors *Fas* and *Fas L* on the thyrocyte surface was reliably lower than in the control group ($p<0.05$), which did not depend on the polymorphic variants of the *BCL-2* gene. However, the indices of Ki-67 proliferation and apoptosis due to the proteins-oncosuppressors *Bcl-2* and *p53* in patients with thyroid pathology, with a density of corresponding proteins within the cell, were reliably higher than those in the control group ($p<0.05$).

Univariate analysis of variance confirmed the association of the promoter of *BCL-2* (*rs*17759659) gene with the number of cells expressing *Bcl*-2 (F=7.25, p<0.001), *p*53 (F=10.58, p<0.001), *Fas* (F=25.33, p<0.001), *Fas L* (F=7.18, p=0.001), *Ki*-67 (F=3.60. p=0.03) and with the density of the receptors *Fas L* (F=9.74, p<0.001) as well as the protein proliferation marker *Ki*-67 (F=13.20. p<0.001) (table 2).

Receptor density and the number of immunoreactive cells of apoptosis and proliferation markers in the thyroid tissue considering the polymorphic variants of CTLA-4 (rs231775) gene are shown in table 3. The density of intracellular protein that regulates the process of Ki-67 proliferation prevailed in the carriers of minor allele G (AG-, GG-genotypes) of the CTLA-4 gene over-AA genotype by 10% (p=0.033) and by11.5% (p=0.046). For the rest of the markers, there were no reliable differences depending on the polymorphism of the CTLA-4 (rs231775) gene. The number of cells with receptors to *Fas, Fas L* and *Ki-67*, as well as the density of intracellular anti-apoptotic proteins p53 and Bcl-2 and proliferation of Ki-67 reliably prevailed the reference values of the control group ($p\leq0.048$-0.001).

Univariate analysis of variance confirmed the association of the promoter of CTLA-4 (rs231775) gene with the number of cells expressing p53 (F=8.35, p<0.001), Fas (F=4.23, p=0.017), Fas L (F=5.61, p=0.005), Ki-67 (F=3.72, p=0.027) and the density of receptors Fas (F=17.17, p=0.001) of anti-apoptotic Bcl-2 (F=3.09, p=0.049) and p53 (F=18.18, p<0.001) and the proliferation marker of Ki-67 protein (F=56.26, p<0.001) (table 4). The receptor density indices and the number of the apoptosis and proliferation cells-markers in the thyroid tissue do not depend directly on the polymorphic variants of the *APO-1/Fas* (*rs*2234767) gene (table 4). The number of the cells with receptors to *Fas*, *Fas L* and Ki-67, as well as the density of intracellular anti-

apoptotic p53 and *Bcl*-2 proteins and proliferation of Ki-67 were reliably higher than in the control group (p≤0.019-0.001).

Univariate analysis of variance (table 4) confirmed the association of promoter of the APO-1 / Fas (rs2234767) gene with the number of cells expressing Fas L (F=8.37, p=0.005) and the density of receptors Fas (F=115.28, p<0.001) and intracellular protein p53 (F=10.62, p=0.001).

Table 2. Density of the receptors and the number of the cells in the markers of apoptosis and proliferation in the thyroid tissue according to the polymorphic variants of the *BCL-2* (*rs*17759659) gene.

Indices	Control, n=36	Gene *BCL-2* genotypes in patients		
		AA, n=10	*AG*, n=110	***GG*, n=5**
Fas cells, %	0.79±0.04	23.28±2.30 p<0.001	18.24±3.89 p<0.001	**28.58±0.55 p<0.001 p_{AA}=0.043 p_{AG}=0.018**
Density of Fas receptors, s.u.	13.82±0.40	6.75±1.25 p<0.001	7.38±1.12 p<0.001	**6.45±0.95 p<0.001**
Fas L cells, %	3.85±0.16	11.93±1.71 p=0.003	10.57±1.34 p=0.002	**12.14±1.45 p=0.002**
Density of Fas L receptors, s.u.	11.13±0.85	7.57±0.96 p=0.009	8.29±0.64 p=0.009	**7.34±0.39 p=0.005**
Total number of p53 cells, %	64.14±1.89	67.79±1.27	59.47±7.0	**68.02±1.52**
Density of p53 protein, (total), s.u.	1.41±0.05	3.46±0.93 p=0.035	3.86±0.58 p=0.004	**3.60±0.94 p=0.028**
Ki-67 cells,%	1.16±0.05	4.26±0.53 p=0.001	3.73±0.81 p=0.001	**4.46±1.40 p=0.026**
Density of Ki-67protein, s.u.	1.20±0.07	1.77±0.18 p=0.006	2.11±0.22 p=0.005	**1.88±0.24 p=0.012**
Bc1-2 cells,%	73.05±1.35	80.66±2.99 p=0.027	78.22±2.44 p=0.055	**81.23±3.47 p=0.037**
Density of Bc1-2 protein, s.u.	**3.86±0.16**	**7.18±1.57 p=0.043**	**6.62±1.07 p=0.013**	**7.40±1.49 p=0.026**

Notes: 1.– reliability of index differences compared to those in the control group; p_{AA} – reliability of index differences with carriers of AA-genotype; p_{AG} – reliability of index differences with carriers of AG-genotype.

Table 3. The density of receptors and the number of cells in the markers of apoptosis and proliferation in the thyroid tissue according to the polymorphic variants of the *CTLA-4* (*rs*231775) gene.

Indices	Control, n=36	Genotypes of the *CTLA-4* gene in patients		
		AA, n=59	*AG*, n=62	***GG*, n=4**
Fas cells, %	0.79±0.04	18.62±4.20 p<0.001	18.90±4.02 p<0.001	**12.81±1.25 p<0.001**
Density of Fas receptors, s.u.	13.82±0.40	7.48±1.32 p=0.003	7.10±1.80 p=0.007	**10.12±1.05 p=0.01**
Fas L cells, %	3.85±0.16	10.64±1.40 p=0.003	10.81±1.26 p=0.002	**8.52±1.18 p=0.006**
Density of Fas L receptors, s.u.	11.13±0.85	8.20±0.61 p=0.009	8.15±0.57 p=0.004	**8.19±0.47 p=0.005**
Total number of p53 cells, %	64.14±1.89	61.46±4.39	58.39±5.26	**65.03±2.90**
Density of p53 protein, (total), s.u.	1.41±0.05	3.71±0.41 p=0.002	4.01±0.35 p<0.001	**3.03±0.56 p=0.008**
Ki-67cells,%	1.16±0.05	3.71±0.62 p=0.005	3.89±0.63 p=0.004	**3.09±0.77 p=0.019**
Density of Ki-67protein, s.u.	1.20±0.07	2.0±0.08 p<0.001	2.20±0.11 p<0.001 p_{AA}=0.033	**2.23±0.08 p<0.001 p_{AA}=0.046**
Bc1-2 cells,%	73.05±1.35	78.49±3.24	78.40±2.36	**77.21±4.82**
Density of Bc1-2 protein, s.u.	**3.86±0.16**	**6.61±0.60 p=0.004**	**6.79±0.64 p=0.003**	**6.08±1.0 p=0.037**

Notes: 1. TG – Thyroid gland; 2. p – reliability of differences between the indices as compared to those in the control group; p_{AA} – reliability of differences between the indices as compared to those in the carriers of the AA-genotype; p_{AG} – reliability of differences between the indices as compared to those in the carriers of the AG-genotype.

Based on the frequency of increasing (moderate, significant) or decreasing the number of cells and the density of receptors expressing the markers of apoptosis and proliferation considering polymorphism of the APO-1 / Fas (rs2234767) gene we established the trends similar to those described in tables 3.24 and 3.26 with the frequency difference 3.08 and 3.60 fold (p<0.001), without reliable differences according to polymorphic variants of the *Fas* (rs2234767) gene.

Analysis of markers of apoptosis and proliferation, as the risk factors of the studied thyroid pathology showed that high compensatory increase in cells in the biopsy expressing Fas, Fas L and Ki-67 and a moderate increase in cells with Bcl-2 with significant decrease in the density of receptors on the cell surface of Fas and Fas L and an increase in the density within the cell of the anti-apoptotic Bcl-2 protein increases the risk of thyroid pathology (AIT and TA) by 2.79 and 9 times in the carriers of

AG- and, in particular, AA-genotypes of the BCL-2 (rs17759659) gene, respectively (OR=7.80 and OR=81,0; p<0.001) (table 5). On the other hand, a significant increase of the proliferation Ki-67 protein density by reducing the number of cells containing p53 oncosuppression protein (significant, >50 percentiles) and a moderate reduction of Bcl-2 protein (≤50 percentiles) is a protection factor and make the chances of AIT and TA occurrence the lowest in the surveyed population of the Northern Bukovyna residents regardless of BCL-2 gene genotypes (OR=0.01; 95% CI OR: 0.001-0.23 for AA-genotype and OR=0.13; 95% CI OR: 0.07-0.23 for AG-genotype, respectively, p<0.001).

Table 4. The density of receptors and the number of cells in the markers of apoptosis and proliferation in the thyroid tissue according to the polymorphic variants of the *APO-1/Fas* (*rs*2234767) gene.

Indices	Control, n=36	Genotypes of the *APO-1/Fas* gene in patients	
		AG, n=23	***GG*, n=102**
Fas cells, %	0.79±0.04	17.70±4.35 p<0.001	**18.75±4.25 p<0.001**
Density of Fas receptors, s.u.	13.82±0.40	7.10±1.80 p=0.007	**10.12±1.05 p=0.009**
Fas L cells, %	3.85±0.16	7.95±1.17 p=0.008	**7.25±1.02 p=0.009**
Density of Fas L receptors, s.u.	11.13±0.85	10.30±1.42	**10.73±1.33**
Total number of p53 cells, %	64.14±1.89	60.83±4.59	**60.09±4.83**
Density of p53 protein, (total), s.u.	1.41±0.05	3.58±0.41 p=0.002	**3.87±0.38 p=0.001**
Number of Ki-67cells, %	1.16±0.05	3.72±0.66 p=0.006	**3.79±0.63 p=0.004**
Density of Ki-67protein, s.u.	1.20±0.07	2.02±0.20 p=0.006	**2.09±0.21 p=0.005**
Number of Bc1-2 cells, %	73.05±1.35	78.34±2.63	**78.42±1.81 p=0.02**
Density of Bc1-2 protein, s.u.	**3.86±0.16**	**6.49±0.63 p=0.005**	**6.70±0.58 p=0.003**

Notes: 1.– reliability of differences between the indices as compared to those in the control group; p_{AG} – reliability of differences between the indices as compared to those in the carriers of the AG-genotype.

Table 5. Polymorphic variants of the *BCL-2* (*rs*17759659) gene as the risk factors of apoptosis and proliferation in the thyroid tissue.

***BCL-2* gene**		***RelR***	***OR***	***95%CI RR***	***95%CI OR***	**p**
Fas, Fas L and Ki-67cells, % (significant increase)	*AA*	9.0	81.0	1.38-58.44	4.36-1504.5	<0.001
	AG	2.79	7.80	2.0-3.89	4.28-14.21	<0.001
Density of *Fas and Fas L* receptors, s.u. (*sifnificant increase*)	*AA*	9.0	81.0	1.38-58.44	4.36-1504.5	<0.001
	AG	2.79	7.80	2.0-3.89	4.28-14.21	<0.001
Density of *Ki*-67protein, s.u. (*significant increase*)	*AA*	0.11	0.01	0.02-0.72	0.001-0.23	<0.001
	AG	0.36	0.13	0.26-0.50	0.07-0.23	<0.001
Total number of p53cells, % ((significant increase)	*AA*	0.11	0.01	0.02-0.72	0.001-0.23	<0.001
	AG	0.36	0.13	0.26-0.50	0.07-0.23	<0.001
Bc1*-2 cells, % (*moderate decrease)	*AA*	0.11	0.01	0.02-0.72	0.001-0.23	<0.001
	AG	0.36	0.13	0.26-0.50	0.07-0.23	<0.001
Bc1*-2 cells, % (*moderate increase)	*AA*	9.0	81.0	1.38-58.44	4.36-1504.5	<0.001
	AG	2.79	7.80	2.0-3.89	4.28-14.21	<0.001
Density of *Bc1*-2 protein, s.u. (*significant increase)*	*AA*	9.0	81.0	1.38-58.44	4.36-1504.5	<0.001
	AG	2.79	7.80	2.0-3.89	4.28-14.21	<0.001

Note. *RelR* - Relative Risk; *OR* -Odds Ratio; *95%CI RR, OR* – 95% confidence interval of Risk Ratio, Odds Ratio

We have found that the analyzed markers of thyroid tissue apoptosis and proliferation (high content of cells in the biopsy expressing Fas, Fas L, Ki-67, Bcl-2, a significant decrease of the Fas and Fas L receptors density on the cell surface and a high increase of the Bcl-2 protein density increase the risk of thyroid pathology (AIT and TA) by 3.92 times in AA genotype carriers of the CTLA-4 gene (OR=15,34; 95% CI OR: 6,26-37,60; p<0.001) (table 6) and by 2.44 times in AG-genotype patients of the CTLA-4 gene (OR=5,98; 95% CI OR: 2,75-12,98; p<0.001) and by 3,08 times in homozygous wild G-allele carriers of the APO-1 / Fas (rs2234767) gene (table 7) and by 3,60 times in AG-genotype patients of the above-mentioned gene (OR=9,49; 95% CI OR: 5,01-17,96 and OR=12,96; 95% CI OR: 3,19-52,62; p<0.001), respectively.

The factors which decrease the likelihood of NGAIT occurrence in the examined patients regardless of the genotypes of the *CTLA*-4 (*rs*231775) and *APO-1/Fas* (*rs*2234767) genes are (tables 6, 7): high compensatory increase in the Ki-67 protein proliferation density and reduction of the cells containing the proteins p53 or Bcl-2 (OR=0.07-0.17; 95% CI OR: 0.03-0.36; p<0.001, and OR=0.08-0.11; 95% CI OR: 0.02-0.31; p<0.001, respectively).

Thus, the NGAIT patients with compensatory increased number of immunoreactive cells expressing Ki-67 and this protein density, the most strongly associated with the CTLA-4 gene polymorphic site (F=56,26; p<0.001) and almost 4 times less with the BCL-2 gene promoter (F=13,20; p<0.001) and is reliably higher only in the minor G-allele carriers of CTLA-4 gene by 10% (p=0.033) and 11.5% (p=0.046), indicating the maintenance of the stored follicular thyroid epithelium regeneration, especially in G-allele carriers.

High concentrations of cells expressing Fas, Fas L, Ki-67, Bcl-2 (>50 percentiles) in thyroid biopsies, accompanied by Fas and Fas L receptors density reduction on the cell surface (<50

percentiles) and high protein Bcl-2 density growth, increase the NGAIT risk in observed population 2.79 in AG- and particular AA-genotype carriers of BCL-2 gene (rs17759659) (OR=7,80 and OR=81,0; p<0.001, respectively); 2.44 times in the AG- and especially AA-genotypes carriers of the CTLA-4 gene (OR=15,34; 95% CI OR: 6,26-37,60; p<0.001 and OR=5,98; 95% CI OR: 2,75-12,98; p<0.001); by 3.08 times in the GG- and AG genotype patients of the *Fas (rs2234767)* gene (OR=9,49; 95% CI OR: 5,01-17,96 and OR=12.96; 95% CI OR: 3,19-52,62; p<0.001), respectively.

Protection factors that reduce the NGAIT likelihood in the surveyed population of the Northern Bukovyna, regardless of the analyzed genes polimorpic variants, include compensatory increase the Ki-67 proliferation protein density in the thyrocyte (>50 percentiles) and reduction of the cells containing p53 or Bcl-2 proteins (OR=0.01-0.17; 95% CI OR: 0.001-0.36; p<0.001 and OR=0.07-0.13; 95% CI OR: 0.02-0.31; p<0.001, respectively).

Table 6. Polymorphic variants of the *CTLA-4* (*rs*231775) gene as risk factors of apoptosis and proliferation in the thyroid tissue.

Indices / genotypes of the *CTLA-4* gene		*RelR*	*OR*	*95%CI RR*	*95%CI OR*	p
Number of *Fas, Fas L* i *Ki*-67 cells, % (*sifnificant increase*)	*AA*	3.92	15.34	2.33-6.60	6.26-37.60	<0.001
	AG	2.44	5.98	1.61-3.72	2.75-12.98	<0.001
Density of *Fas and Fas L* receptors, s.u. (*significant decrease*)	*AA*	3.92	15.34	2.33-6.60	6.26-37.60	<0.001
	AG	2.44	5.98	1.61-3.72	2.75-12.98	<0.001
Density of *Ki*-67protein, y.o. (*significant increase*	*AA*	0.25	0.07	0.15-0.43	0.03-0.16	<0.001
	AG	0.41	0.17	0.27-0.62	0.08-0.36	<0.001
Total number of p53 cells, % (*significant decrease*)	*AA*	0.25	0.07	0.15-0.43	0.03-0.16	<0.001
	AG	0.41	0.17	0.27-0.62	0.08-0.36	<0.001
Number of *Bc1*-2 cells, % (*moderate decrease*)	*AA*	0.25	0.07	0.15-0.43	0.03-0.16	<0.001
	AG	0.41	0.17	0.27-0.62	0.08-0.36	<0.001
Number of *Bc1*-2 cells, % (*moderate increae*)	*AA*	3.92	15.34	2.33-6.60	6.26-37.60	<0.001
	AG	2.44	5.98	1.61-3.72	2.75-12.98	<0.001
Density of *Bc1*-2 protein, s.u. (*significant increase*)	*AA*	3.92	15.34	2.33-6.60	6.26-37.60	<0.001
	AG	2.44	5.98	1.61-3.72	2.75-12.98	<0.001

Note. *RelR* - Relative Risk; *OR* -Odds Ratio; *95%CI RR, OR* – 95% confidence interval of Risk Ratio, Odds Ratio

Table 7. Polymorphic variants of the *APO-1/Fas* (*rs*2234767) gene as risk factors of apoptosis and proliferation in the thyroid tissue.

Indices / genotypes of the *APO-1/Fas* gene		*RelR*	*OR*	*95%CI RR*	*95%CI OR*	p
Number of *Fas, Fas L* i *Ki*-67cells, % (*significant increase*)	*AG*	3.60	12.96	1.61-8.05	3.19-52.62	<0.001
	GG	3.08	9.49	2.15-4.41	5.01-17.96	<0.001
Density of *Fas i Fas L* receptors, s.u. (significant decrease*)*	*AG*	3.60	12.96	1.61-8.05	3.19-52.62	<0.001
	GG	3.08	9.49	2.15-4.41	5.01-17.96	<0.001
Density of *Ki*-67protein, s.u. (significant increase)	*AG*	0.28	0.08	0.12-0.62	0.02-0.31	<0.001
	GG	0.32	0.11	0.23-0.46	0.06-0.20	<0.001
Total number of p53 cells, % (significant decrease)	*AG*	0.28	0.08	0.12-0.62	0.02-0.31	<0.001
	GG	0.32	0.11	0.23-0.46	0.06-0.20	<0.001
Number of *Bc1*-2 cells, % (moderate decrease)	*AG*	0.28	0.08	0.12-0.62	0.02-0.31	<0.001
	GG	0.32	0.11	0.23-0.46	0.06-0.20	<0.001
Number of *Bc1*-2 cells, % (moderate decrease)	*AG*	3.60	12.96	1.61-8.05	3.19-52.62	<0.001
	GG	3.08	9.49	2.15-4.41	5.01-17.96	<0.001
Density of *Bc1*-2 protein, s.u. (significant increase)	*AG*	3.60	12.96	1.61-8.05	3.19-52.62	<0.001
	GG	3.08	9.49	2.15-4.41	5.01-17.96	<0.001

Note. *RelR* - Relative Risk; *OR* -Odds Ratio; *95%CI RR, OR* – 95% confidence interval of Risk Ratio, Odds Ratio

4. CONCLUSIONS

Thus, patients with NGAIT activate several links of a programmed thyrocyte killing where Fas-induced apoptosis prevails and is the most associated with the promoter of the *BCL-2* (*rs*17759659) gene (F=25,33; p<0,001) and about 6 times less with the promoter of the *CTLA*-4 (*rs*231775) gene (F=4,23, p=0,017), due to the pronounced expression of *Fas* i *Fas L* on the cellular surface in the areas of the lymphoid infiltration and destruction of thyrocytes (more pronounced in the carriers of *GG*-genotype of the *BCL*-2 gene– by18,54% (p_{AA}=0,043) and 36,18% (p_{AG}=0,018) respectively), which indicates the initiation of apoptosis external way through the caspase mechanism (effector caspases 8).

2. An increased expression of Bcl-2 in the thyroid lymphocytes of patients with AIT and TA is associated exclusively with the promoter region of the BCL-2 (rs17759659) gene according to the results of univariate analysis of variance (F = 7,25, p <0,001), without a clear dependence on certain polymorphic variants, with unreliable changes in the expression of p53 protein (it is also associated with the polymorphic site of BCL-2, F = 10,58; p <0,001), indicating a slight control of apoptosis (despite a compensatory increase in the density of p53

inside the thyrocyte, which, although connected with the promoter regions of CTLA-4 (F = 18,18, p <0,001) and APO-1 / Fas (F = 10,62, p = 0,0 01) genes, does not affect significantly (p> 0.05) the expression of this protein) with a possible extension of the cell survival time and insufficient removal from the pool of the cells that are potentially oncogenic, which supposedly can contribute to carcinogenesis.

The patients with NGAIT have compensatory increased number of immunoreactive cells expressing Ki-67 and the density of this protein, which is most strongly associated with polymorphic site of the CTLA-4 gene (F = 56,26; p <0,001) and almost 4 times less with the promoter of the BCL-2 gene (F = 13,20; p <0,001) and is reliably higher only in the G-owners of minor allele gene CTLA-4 by 10% (p = 0.033) and 11.5% (p = 0.046), indicating the maintaining of the stored follicular thyroid epithelium regeneration, especially in the carriers of this allele.

High concentrations of the cells expressing Fas, Fas L, Ki-67, Bcl-2 (> 50 percentiles) in the thyroid biopsy, accompanied bya reduction of the density of Fas and Fas L receptors on the cell surface (<50 percentiles) and high growth of the protein Bcl- 2 density increase the risk of NGAIT: by 2.79 and 9 times AG-media and, in particular, AA-genotype BCL-2 gene (rs17759659), respectively (OR = 7,80 and OR = 81,0; p <0.001); by 2.44 and 3.92 times in the AG carriers and especially AA genotypes of the CTLA-4 gene (OR = 15,34; 95% CI OR: 6,26-37,60; p <0.001 and OR = 5,98 ; 95% CI OR: 2,75-12,98; p <0.001); by 3.08 and 3.60 times in the homozygous owners of the main G-allele and the AG genotype of the *APO-1 / Fas (rs2234767)* gene (OR = 9,49; 95% CI OR: 5,01-17,96 and OR = 12.96; 95% CI OR: 3,19-52,62; p <0.001), respectively.

Protection factors that reduce the likelihood of NGAIT in the surveyed population of the Northern Bukovyna residents, regardless of genotypes of the analyzed genes, include compensatory increase in the density of proliferation protein in the Ki-67 thyrocyte (> 50 percentiles) and reduction of the cells containing p53 or Bcl- 2 proteins (OR = 0,01-0,17; 95% CI OR: 0,001-0,36; p <0.001 and OR = 0,07-0,13; 95% CI OR: 0,02-0,31; p <0.001, respectively).

5. REFERENCES

1. Larson, S.D.; Jackson, L.N.; Riall, T.S.; Uchida, T.; Thomas, T.S.; Qiu, S.; Evers, B.M. Increased incidence of well-differentiated thyroid cancer associated with Hashimoto thyroiditis and the role of the PI3k/Akt pathway. *J Am Coll Surg*. **2007**, *204*, 764-73, https://dx.doi.org/10.1016%2Fj.jamcollsurg.2006.12.037.
2. Effraimidis, G.; Wiersinga, W.M. Mechanisms in endocrinology: autoimmune thyroid disease: old and new players. *Eur J Endocrinol*. **2014**, *170*, R241-52, https://doi.org/10.1530/EJE-14-0047.
3. Eschler, D.C.; Hasham, A.; Tomer, Y. Cutting edge: the etiology of autoimmune thyroid diseases. *Clin. Rev. Allergy Immunol.* **2011**, *13*, 33–39, https://doi.org/10.1007/s12016-010-8245-8.
4. Brix, T.H.; Hegedus, L. Twin studies as a model for exploring the aetiology ofautoimmune thyroid disease. *Clin Endocrinol (Oxf).* **2012**, *76,* 457-464, https://doi.org/10.1111/j.1365-2265.2011.04318.x.
5. Tsyganenko, O.S.; Voroschuk, R.S. Immunomorfological reaction in the thyroid tissue in patients with autoimmune thyroiditis in combination with nodular goiter. *Arta Medica. Nicholas Anestiadi, Tenth Congress of the Association of Surgeons of Moldova: Chisinau*. **2007**, *4*. 51-52.
6. Sheremet, M.I.; Sydorchuk, L.P.; Shidlovskyi, V.O.; Bedenyuk, A.D. Research of prognostic markers of proliferation and apoptosis in patients with nodular goiters combined with autoimmune thyroiditis. *Archives of the Balkan Medical Union* **2016**, *51*, 488-491.
7. Sheremet, M.I.; Sydorchuk, L.P.; Shidlovskyi, V.O.; Bedenyuk A.D.; Pashkovska, M.O.; Leonova, M.O.; Chorna, O.O.; Stankova, N.I.; Rybak, O.V. New prognostistic markers of nodular forms of goiter combined with autoimmune thyroiditis. *Journal of Education, Health and Sport*. **2017**, *7,* 475-482, http://dx.doi.org/10.5281/zenodo.399322.
8. Sydorchuk, L.P.; Sydorchuk, A.R.; Sheremet, M.I.; Sydorchuk, R.I.; et al. Cytokines cascade changes in patients with rheumatoid arthritis depending on endothelial no-synthase (T-786C) genes polymorphism. *Archives of the Balkan Medical Union* **2017**, *52,* 32-38.
9. Navratil, J.S.; Ahearn, J.M. Apoptosis and autoimmunity: complement deficiency and systemic lupus erythematous revisited. *Curr Rheumatol Rep.* **2000**, *2*, 32-38, https://doi.org/10.1007/s11926-996-0066-7.
10. Giordano, C.; Stassi, G.; De Maria, R.; Todaro, M.; Richiusa, P.; Papoff, G.; Ruberti, G.; Bagnasco, M.; Testi, R.; Galluzzo, A. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. *Science* **1997**, *275*, 960-963, https://doi.org/10.1126/science.275.5302.960.
11. Lydon, A.; Martyn, J.A. Apoptosis in critical illness. Int. *Anesthesiol. Clin*. **2003**, *N 41*, 5-77, https://doi.org/10.1007/978-0-387-77383-4_35.
12. Emma, R.; Dorris, P.S.; O'Leary, J.J.; Orla, S. MIR141 expression differentiates Hashimoto thyroiditis from PTC and benign thyrocytes in Irish archival thyroid tissues. *Front Endocrinol (Lausanne)* **2012**, *3,* 102, https://doi.org/10.3389/fendo.2012.00102.
13. Kazakov, S.P.; Kushlinsky, N.Y. The investigation of CD 95, p53, bcl-2 and Ki-67 markers in autoimmune thyroid pathology patients. First Joint Meeting of European National Societies of Immunology Under the auspices of EFIS and 16th European Congress of Immunology: ECI. 2006 Sept. 6-9; Paris, France: 547.
14. Tomer, Y.; Huber, A. The etiology of autoimmune thyroid disease: a story of genes and environment. *J Autoimmun.* **2009**, *32,* 231-9, https://doi.org/10.1016/j.jaut.2009.02.007.
15. McLachlan, S.M.; Rapoport, B. Breaking tolerance to thyroid antigens: changing concepts in thyroid autoimmunity. *Endocr Rev*. **2014**, *35*, 59-105, https://doi.org/10.1210/er.2013-1055.
16. Ganchevska, P.; Murdjev, K.; Sarafian, V. Expression of proliferative antigens in human thyroid diseases. *Trakia Journal of Sciences* **2004**, *2*, 16–20. http://www.uni-sz.bg
17. Dong, Y.H.; Fu, D.G. Autoimmune thyroid disease: mechanism, genetics and current knowledge. *Eur Rev Med Pharmacol Sci.* **2014**, *18*, 3611-3618.
18. Gözü, H.I.; Özçelik, S.; Aloğlu, M.; Şahin, A.; Temiz, S.; Dayan, A.; Cengiz, H.; Tütüncü, Y.; Bircan, R. Is the TSHR D727E polymorphism a genetic predisposition for multinodular goiter in the Turkish population? *Genet Mol Res.* **2016**, *15*, 385–90, https://doi.org/10.4238/gmr.15038504.
19. Kochetova, O.V.; Gaynullina, M.K.; Viktorova, T.V. DIO2, TPO, CYP1A1 AND CYP1A2 gene polymorphism in women

with thyroid disease. *Gig Sanit.* **2014**, *3*, 52–56. PMID: 25306702

20. Lee Y.H.; Choi, S.J.; Ji, J.D.; Song, G.G. CTLA-4 and TNF-α promoter-308 A/G polymorphisms and ANCA-associated vasculitis susceptibility: a meta-analysis. *Mol Biol Rep.* **2012**, *39,* 319-326, https://doi.org/10.1007/s11033-011-0741-2.

21. Nikitin, Y.P.; Rymar, O.D.; Maksimov, V.N.; Simonova, G.; Zankina, M.; Mustafina, S.V.; Sherbacova, L.; Chernova, N.; Voevoda, M.I. Association of the T-cell regulatory Ggene CTLA-4 with suscep-tibility to autoimmune thyroid disease in population of Novosibirsk. *Clinical and experimental thyroidology* **2008**, *4*, 41-45 [in Russian], https://doi.org/10.14341/ket20084441-45.

22. Man-Man, L.; Qian-Ling, Y.; Chen-Chen, F.; Jie, Y.; Tao, Z.; Jing, L.; Rui-Xue, L.; Hai-Feng, P.; Hui, Y.; Dong-Qing, Y. Association of FAS gene polymorphisms with systemic lupus erythematosus: A case-control study and meta-analysis. *Experimental and Therapeutic Medicine* **2012**, *4*, 497-502, https://dx.doi.org/10.3892%2Fetm.2012.625.

23. 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*, 179-188, https://doi.org/10.5281/zenodo.45327.