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ASSOCIATIONS OF BCL-2 (RS17759659), CTLA-4 (RS231775), APO-1/FAS (RS2234767) GENES POLYMORPHISMS WITH ACTIVITY OF PROLIFERATION AND APOPTOSIS IN THYROID TISSUE OF PATIENTS WITH NODULAR FORMS OF GOITER COMBINED WITH AUTOIMMUNE THYROIDITIS AND THYROID ADENOMA

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Abstract

The study of apoptosis and proliferative activity in the thyroid gland (TG) tissue of patients with nodular goiter and autoimmune thyroiditis (NGAIT) and thyroid adenoma (TA) is based on the expression/density of Fas/FasL, BCL-2, p53, and Ki-67 markers assessment depending on the genetic polymorphisms of BCL-2 (rs17759659), CTLA-4 (rs231775) and APO-1/Fas (rs2234767) genes.

Several mechanisms of thyroid cells' programmed killing are activated in NGAIT and TA with domination of Fas-induced apoptosis, which strongly associates with the BCL-2 gene's (rs17759659) promoter ($F=25.33$; $p<0.001$) and almost six fold weaker associates with the CTLA-4 gene's (rs231775) promoter ($F=4.23$, $p=0.017$). Factors that decrease the

likelihood of NGAIT and TA regardless of the CTLA-4 (rs231775) and APO-1/Fas (rs2234767) genes' genotypes are the high Ki-67 density and reduction of cells containing p53 or BCL-2 proteins (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). High expression of surface Fas and FasL in lymphoid infiltration and destruction of thyroid cells (stronger in GG-genotype carriers of the BCL-2 gene by 18.54% (p_{AA}=0.043) and 36.18% (p_{AG}=0.018), respectively) indicates the initiation of the external pathway of apoptosis through the caspase mechanism (effector caspase- 8).

Key words: nodular goiter with autoimmune thyroiditis, thyroid adenoma, proliferation, apoptosis, APO-1 / FAS, CTLA-4 and BCL-2 genes polymorphisms.

1. Introduction

Multinodular goiter is the most common disorder of the thyroid gland because of the genetic heterogeneity of follicular cells and apparent acquisition of new cellular festures that become inheritable [1]. The etiology of nodular goiters is not completely understood. Age-related increase in thyroid weight and nodularity may be secondary to iodine deficiency rather than an inherent ageing of thyroid gland [2, 3]. Endemic goiters secondary to iodine deficiency are associated with increased formation of hyperplastic nodules partly because of overstimulation of thyroid gland with thyroid stimulating hormone [4]. Another cause of nodular goiter is Hashimoto’s disease [5-7]. Autoimmune thyroiditis (AIT) is a chronic autoimmune disorder characterized by lymphocytic inflammation of thyroid gland. The triggers for this autoimmune response appear to be both humoral and cellular, with complex etiology involving genetic and environmental factors [8]. Individuals with Hashimoto's thyroiditis have an increased risk for developing other autoimmune disorders, including rheumatoid arthritis, Addison's disease, type 1 diabetes mellitus, multiple sclerosis, and pernicious anemia [8, 9].

The interaction of Fas with its ligand FasL regulates a number of physiologic and pathologic processes of cell death. Triggering of Fas contributes to the regulation of immune response and tissue homeostasis, as well as to the immunologic clearance of virus or tumor cells. The destructive processes overcome the potential capacity of thyroid replacement, estimated as about 5- to 10-fold in a lifetime [10]. Apoptosis has been occasionally observed in histologic sections of normal thyroid. However, apoptotic cell death is abnormally accelerated during the pathologic phases, leading to clinical hypothyroidism in AIT. IL-1P, abundantly produced in TG, induces Fas expression in normal thyrocytes, and crosslinking of

Fas results in massive thyrocytes apoptosis. The ligand for Fas is shown to be constitutively expressed in both normal and AIT thyrocytes and is able to kill Fas-sensitive targets. Exposure to IL-induced thyrocytes apoptosis, which is prevented by antibodies that block Fas, suggesting that IL-induced Fas expression serves as a limited factor for thyrocytes destruction. Thus, Fas-FasL interactions within AIT thyrocytes may contribute to clinical hypothyroidism [10]. Majority of authors focused on the molecular markers of apoptosis and proliferation in the thyroid gland cancer and toxic goiter [11-13]. Variations in several genes have been studied as possible risk factors for Hashimoto thyroiditis. Most of the genetic variations that have been discovered are thought to have a moderate impact on an individual overall risk of developing this condition [14-22].

The aim of this study is to find possible associations of BCL-2 (rs17759659), CTLA-4 (rs231775), APO-1/Fas (rs2234767) genes' polymorphisms with activity of proliferation and apoptosis (expression/density of Fas/FasL, BCL-2, p53, and Ki-67) in thyroid tissue of patients with nodular forms of goiter combined with autoimmune thyroiditis and thyroid adenoma.

2. Material and methods

2.1. Subjects

One hundred and twenty-five women with NGAIT underwent examination during 2013-2016, based on Chemivtsi Regional Hospital, Ukraine. The age of patients ranged from 23-72 years (mean age (±SD) 43.7±7.11). Diagnosis is primarily based on clinical and laboratory examinations (thyroid peroxidase antibodies (TPOAb) - 60-250 U/ml, thyroglobulin antibodies (TgAb) - 60-500 U/ml, thyroid-stimulating hormone (TSH) - 4.10 IU/l, thyroid sonography, and histologic confirmation after surgery). Control group included 25 healthy female donors of respective age and socio-economic status.

2.2. Tissue samples collection and evaluation

During surgery, thyroid tissue was collected for immunohistochemical studies no later than 30 minutes after the intervention. In patients with TA, the unchanged tissue of the TG lobe and adenomatous tissue was taken, too. Additionally, in AIT patients the tissue from both lobes and from the isthmus was taken. Pieces of tissue weighing 100-300 mg were freezed and immediately transported and cut into 4-6 pieces weighing 50-70 mg each.

Tissue bioptates underwent homogenization with further thyrocytes' cell suspension coloring with monoclonal antibodies (MAbs) to membrane receptors and intracellular proteins. Following MAbs panels were used: 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 FAS 18; Mouse Anti-Human CD95L Monoclonal Antibody, Unconjugated, Clone NOK-1 (Dako Denmark A/S).

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

While counting cells we evaluated proliferation and apoptosis indices in study areas, using gating (Fig. 1), selecting the window with cells under 25 microns.

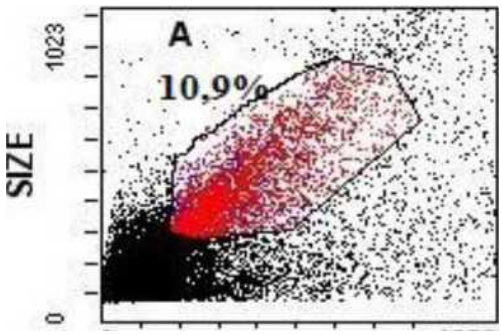

Fig. 1: Histogram of the thyroid tissue heterogeneous suspension with limited gating area (A)

The number of cells, density of superficial (Fas, FasL) and intracellular proliferation, and apoptosis markers (Ki-67, BCL-2, p53) were determined. Phenotyping was performed on flow cytofluorimeter (FACS Calibur, BD Biosciences) counting 100,000 events in each sample, calculating the relative number of cells, and measuring the density/expression of receptors (proteins) in cells or groups of cells. Digital data (histograms) were analyzed in CXP ver. 2.2 software.

2.3. Genotyping of SNPs

DNA extraction was performed using Thermo Scientific Gene JET Genomic DNA Purification kit (#K0721, Thermo Fisher Scientific), with overnight incubation with Proteinase K for complete lysis of the cells. Purified DNA was diluted in Elution Buffer and the quality control was performed on Nano drop 2000C. Only samples that yield at least 15 ng/^l and had A (260/280) between 1.7 and 2.0 were used for genotyping. The DNA was liquated; the stock was stored at -20^{o}C, one aliquot at +4^{o}C until use. All samples were diluted with Nuclease-free water to 2 ng/^l for normalization purpose. TaqMan technique was applied

to genotype selected SNPs. Reference SNP ID numbers from dbSNP were used for polymorphism notation. TaqMan SNP Genotyping Assays (40*) (4351379, Thermo Fisher Scientific) were used for the testing (table 1).

Table 1: Analyzed SNP context sequences

SNP	Assay ID*	Context Sequence
rs231775 (CTLA4)	C 2415786 20	GCACAAGGCTCAGCTGAAC-CTGGCT[A/G]CCAGGACCTGGCCCTGCACTCTCCT
rs17759659 (BCL-2)	C33628167_10	TCTTCTTACCAAAGATTCACAA- TAC [A/G] GTGTTGATGGGAACGTGACCTAGTT
rs2234767 (Fas)	C12123966_10	CAGAGTGT GT GCACAAGGCTGG-CAC[A/G]CCCAGGGTCTTCCTCATGGCACTAA

Reaction volume was 5 ^l and included: 2.5 ^l of TaqMan Genotyping Master Mix (20x) (4371355, Thermo Fisher Scientific), 0.25 ^l of probes solution and 2.25 ^l of DNA solution. Genotyping procedure was done on Quant Studio 6 (Applied Biosystems, Thermo Fisher Scientific), 384-well block. The following amplification program was applied to produce the data:

HOLD (enzyme activation)	10 min	95ºC	
Denaturation	15 sec	92ºC	40*/60**
Annealing / Extension	1 min	60ºC	cycles

Note: * - applied for CTLA4 and Fas associated SNP; ** - for BCL-2 associated SNP.

Quant Studio™ Real-Time PCR software (v. 1.3) was used for data acquisition and analysis.

2.4. Statistical analysis

Statistical analysis was performed using Statistica 7.0 (Stat Soft Inc.) software. Nominal data presented in the form of quantitative and percentages. All variables were normally distributed (Kolmogorov-Smirnov and Shapiro-Wilk tests). Each SNP was tested for deviation from Hardy-Weinberg equilibrium using the Online Encyclopedia for Genetic Epidemiology Studies (http://www.oege.org/software/hardy-weinberg.html). Pearson's criterion (x) was used for the genotypes distribution comparison. Analysis of qualitative data (categorical variables), risk of thyroid pathology development was assessed in 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 (x^2) (df=1). The difference was considered significant at $p<0.05$.

3. Results and discussion

3.1. Association of BCL-2 (rs17759659) gene's polymorphism with proliferation and apoptosis activity in thyroid tissue

Cell numbers and densities of the Fas, FasL, Ki-67, BCL-2, and p53 receptors considering the polymorphic variants of BCL-2 (rs17759659) gene are presented in table 2.

Table 2: Cell numbers and densities of the Fas, FasL, Ki-67, BCL-2, and p53 receptors considering the polymorphic variants of BCL-2 (rs17759659) gene

Indices	Control (morphologically unaltered thyroid tissue), n=25	BCL-2 genotypes		
		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}=^{0018}
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
FasL 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 FasL 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
p53 cells, %	64.14±1.89	67.79±1.27	59.47±7.0	68.02±1.52
Density of p53 protein, 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- 67 protein, 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
BCL-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 BCL-2, 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. TG- thyroid gland; 2. p - 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.

The number of immunoreactive cells expressing surface transmembrane protein Fas is 18.54% (p_{AA}=0.043) and 36.18% (p_{AG}=0.018) higher in homozygous carriers of minor G-allele of the BCL-2 gene than in main A-allele carriers (AA- and AG-genotypes, respectively). Significantly higher number of cells with Fas, FasL receptors, BCL-2, and Ki-67 ($p<0.055$-0.001) observed in patients with thyroid pathology compared to control, whilst the density of Fas and FasL receptors was reliably lower than in control group ($p<0.05$), independent on the polymorphic variants of BCL-2 gene. The proliferation and apoptosis indices (Ki-67, BCL-2, and p53) in patients with thyroid pathology were reliably higher than in control ($p<0.05$).

Univariate statistical analysis confirmed associations of the BCL-2 (rs17759659) gene's promoter with the number of cells expressing BCL-2 ($F=7.25$, $p<0.001$), p53 ($F=10.58$, $p<0.001$), Fas ($F=25.33$, $p<0.001$), FasL ($F=7.18$, $p=0.001$), Ki-67 ($F=3.60$, $p=0.03$), and with the density of the FasL receptors ($F=9.74$, $p<0.001$), as well as the protein proliferation marker Ki-67 ($F=13.20$, $p<0.001$).

3.2. Associations of CTLA-4 (rs231775) gene's polymorphism with proliferation and apoptosis activity in thyroid tissue

Associations of cell numbers and densities of the Fas, FasL, Ki-67, BCL-2, and p53 receptors with the polymorphic variants of CTLA-4 (rs231775) gene are shown in table 3. The density of intracellular protein Ki-67, which regulates the process of proliferation prevailed in the carriers of minor allele G (AG- and GG-genotypes) of the CTLA-4 gene compared to AA-genotype by 10% ($p=0.033$) and 11.5% ($p=0.046$), respectively. There were no reliable differences depending on the polymorphism of the CTLA-4 (rs231775) gene for the rest of the markers. The number of cells with receptors to Fas, FasL, and Ki-67, as well as the densities of p53, BCL-2, and Ki-67 prevailed over the control values ($p<0.048$-0.001).

Univariate statistical analysis confirmed the associations of the CTLA-4 (rs231775) gene's promotor with the number of cells expressing p53 ($F=8.35$, $p<0.001$), Fas ($F=4.23$, $p=0.017$), FasL ($F=5.61$, $p=0.005$), Ki-67 ($F=3.72$, $p=0.027$) and the densities of Fas ($F=17.17$, $p=0.001$), BCL-2 ($F=3.09$, $p=0.049$), p53 ($F=18.18$, $p<0.001$), and Ki-67 proteins ($F=56.26$, $p<0.001$).

Table 3: Cell numbers and densities of the Fas, FasL, Ki-67, BCL-2, and p53 receptors considering the polymorphic variants of CTLA-4 (rs231775) gene

Indices	Control (morphologically unaltered thyroid tissue), n=25	CTLA-4 genotypes		
		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
FasL 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 FasL 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
p53 cells, %	64.14±1.89	61.46±4.39	58.39±5.26	65.03±2.90
Density of p53 protein, 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-67 cells, %	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-67 protein, 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
BCL-2 cells, %	73.05±1.35	78.49±3.24	78.40±2.36	77.21±4.82
Density of BCL- 2, 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 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.

3.3. Associations of APO-1/Fas (rs2234767) gene's polymorphism with proliferation and apoptosis activity in thyroid tissue

The number of cells with receptors to Fas, FasL and Ki-67, as well as the densities of intracellular anti-apoptotic p53 and BCL-2 proteins and Ki-67 were reliably higher than in control group (p<0.019-0.001). Cell numbers figures and densities of the receptors did not associate directly with the polymorphic variants of the APO- 1/Fas (rs2234767) gene (table 4).

Table 4: Cell numbers and densities of the Fas, FasL, Ki-67, BCL-2, and p53 receptors considering the polymorphic variants of APO-1/Fas (rs2234767) gene

Indices	Control (morphologically unaltered thyroid tissue), n=25	APO-1/Fas genotypes	
		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
FasL cells, %	3.85±0.16	7.95±1.17 p=0.008	7.25±1.02 p=0.009
Density of FasL receptors, s.u.	11.13±0.85	10.30±1.42	10.73±1.33
p53 cells, %	64.14±1.89	60.83±4.59	60.09±4.83
Density of p53 protein, s.u.	1.41±0.05	3.58±0.41 p=0.002	3.87±0.38 p=0.001
Ki-67 cells, %	1.16±0.05	3.72±0.66 p=0.006	3.79±0.63 p=0.004
Density of Ki-67 protein, s.u.	1.20±0.07	2.02±0.20 p=0.006	2.09±0.21 p=0.005
BCL-2 cells, %	73.05±1.35	78.34±2.63	78.42±1.81 p=0.02
Density of BCL-2, s.u.	3.86±0.16	6.49±0.63 p=0.005	6.70±0.58 p=0.00no3

Notes: 1. TG - thyroid gland; 2. p - 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.

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

3.4. Prediction of AIT and TA depending on BCL-2 (rs17759659), CTLA- 4 (rs231775), and APO-1/Fas (rs2234767) genes' polymorphisms, proliferation, and apoptosis activity in thyroid tissue

High numbers of Fas, FasL, BCL-2, and Ki-67 cells, combined with decreased density of superficial Fas and FasL receptors, and growth of the anti-apoptotic BCL-2 protein,

increases the risk of thyroid pathology (AIT and TA) by 2.79 and 9 times in AG- and AA-genotypes carriers of the BCL-2 (rs17759659) gene (OR=7.80 and OR=81.0; $p<0.001$), respectively. Combination of highly increased (>50 percentiles) Ki-67 protein density with reduced number of p53 containing cells and moderate reduction of BCL-2 protein (<50 percentiles) decreases the chance of AIT and TA 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, $p<0.001$, respectively).

High content of Fas, Fas L, Ki-67, and BCL-2 expressing cells in biopsy, significant decrease of Fas and FasL receptors' density combined with high BCL-2 density increase the risk of 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; $p<0.001$); by 2.44 times in AG- genotype patients (OR=5.98; 95% CI OR: 2.75-12.98; $p<0.001$); by 3.08 times in homozygous wild G-allele carriers of the APO-1/Fas (rs2234767) gene 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 that decrease the likelihood of AIT and TA regardless of the genotypes of the CTLA-4 (rs231775) and APO-1/Fas (rs2234767) genes are the high Ki- 67 density and reduction of cells containing the proteins p53 or BCL-2 (OR=0.07-17; 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).

4. Conclusion

In AIT and TA patients several mechanisms of thyroid cells' programmed killing are activated with domination of Fas-induced apoptosis, which strongly associates with the BCL-2 gene's (rs17759659) promotor ($F=25.33$; $p<0.001$) and almost 6 times weaker associates with the CTLA-4 gene's (rs231775) promotor ($F=4.23$, $p=0.017$). High expression of surface Fas and FasL in lymphoid infiltration and destruction of thyroid cells (stronger in GG-genotype carriers of the BCL-2 gene by 18.54% ($p_{AA}=0.043$) and 36.18% ($p_{AG}=0.018$), respectively) indicates the initiation of the external pathway of apoptosis through the caspase mechanism (effector caspase- 8).

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

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References

1. S. D. Larson, L. N. Jackson, T. S. Riall et al., "Increased incidence of well-differentiated thyroid cancer associated with Hashimoto thyroiditis and the role of the PI3k/Akt pathway", *Journal of the American College of Surgeons,* vol. 204, no. 5, pp. 764-773, 2007.

2. G. Effraimidis and W. M. Wiersinga, "Mechanisms in endocrinology: autoimmune thyroid disease: old and new players", *European Journal of Endocrinology,* vol. 170, no. 6, R241-52, 2014.

3. D. C. Eschler, A. Hasham, and Y. Tomer, "Cutting edge: the etiology of autoimmune thyroid diseases", *Clinical Reviews in Allergy & Immunology,* vol. 41, no. 2, pp. 190-197, 2011.

4. T. H. Brix and L. Hegedus, "Twin studies as a model for exploring the etiology of autoimmune thyroid disease", *Clinical Endocrinology*, vol. 76, no. 4, pp. 457-464, 2012.

5. H. Lahooti, S. Edirimanne, J. P. Walsh et al., "Single nucleotide polymorphism 1623 A/G (rs180195) in the promoter of the Thyroglobulin gene is associated with autoimmune thyroid disease but not with thyroid ophthalmopathy", *Clinical Ophthalmology*, vol. 11, pp. 1337-1345, 2017.

6. M. I. Sheremet, L. P. Sydorchuk, V. O. Shidlovskyi, and A. D. Bedenyuk, "Research of prognostic markers of proliferation and apoptosis in patients with nodular goiters combined with autoimmune thyroiditis", *Archives of the Balkan Medical Union*, vol. 51, no. 4, pp. 488-491, 2016.

7. M. I. Sheremet, L. P. Sydorchuk, V. O. Shidlovskyi et al., "New prognostistic markers of nodular forms of goiter combined with autoimmune thyroiditis", *Journal of Education, Health and Sport*, vol.7, no. 3, pp. 475-482, 2017.

8. L. P. Sydorchuk, A. R. Sydorchuk, M. I. Sheremet 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*, vol. 52, no. 1, pp. 32-38, 2017.

9. J. S. Navratil and J. M. Ahearn, "Apoptosis and autoimmunity: complement deficiency and systemic lupus erythematous revisited", *Current Rheumatology Reports,* vol. 2, pp. 32-38, 2000.

10. C. Giordano, G. Stassi, R. De Maria et al., "Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis", *Science,* vol. 275, 960-963, 1997.

11. A. Lydon and J. A. Martyn, "Apoptosis in critical illness", *International*

Anesthesiology Clinics, vol. 41, no. 1, pp. 65-77, 2003.

12. R. D. Emma, P. Smyth, J. J. O'Leary, and Orla Sheils, "MIR141 expression differentiates Hashimoto thyroiditis from PTC and benign thyrocytes in Irish archival thyroid tissues", *Frontiers in Endocrinology (Lausanne),* vol. 3, pp.102, 2012.

13. M. Versini, "Thyroid autoimmunity and antiphospholipid syndrome: not such a trivial association", *Frontiers in Endocrinology (Lausanne),* vol. 8, pp.175, 2017.

14. Y. Tomer and A. Huber, "The etiology of autoimmune thyroid disease: a story of genes and environment", *Journal of Autoimmunity,* vol. 32, no. 3-4, pp. 231-239, 2009.

15. S. M. McLachlan and B. Rapoport, "Breaking tolerance to thyroid antigens: changing concepts in thyroid autoimmunity", *Endocrine Reviews,* vol. 35, no. 1, pp. 59-105, 2014.

16. P. Ganchevska, K. Murdjev, and V. Sarafian, "Expression of proliferative antigens in human thyroid diseases", *Trakia Journal of Sciences,* vol. 2, no. 1, pp. 16-20, 2004.

17. Y. H. Dong and D. G. Fu, "Autoimmune thyroid disease: mechanism, genetics and current knowledge", *European Review for Medical and Pharmacological Sciences,* vol. 18, no. 23, pp. 3611-3618, 2014.

18. H. I. Gozu, S. Ozfelik, M. Aloglu et al., "Is the TSHR D727E polymorphism a genetic predisposition for multinodular goiter in the Turkish population?", *Genetics and Molecular Research,* vol. 15, no. 3, pp. 385-390, 2016.

19. O. V. Kochetova, M. K. Gaynullina, and T. V. Viktorova, "DIO2, TPO, CYP1A1 AND CYP1A2 gene polymorphism in women with thyroid disease", *Gigiena i Sanitariia,* no. 3, pp. 52-56, 2014 [in Russian].

20. Y. H. Lee, S. J. Choi, J. D. Ji, and G.G. Song, "CTLA-4 and TNF-a promoter-308 A/G polymorphisms and ANCA-associated vasculitis susceptibility: a meta-analysis", *Molecular Biology Reports,* vol. 39, no. 1, pp. 319-326. 2012.

21. Y. P. Nikitin, O. D. Rymar, V. N. Maksimov et al., "Association of the T-cell regulatory gene CTLA-4 with susceptibility to autoimmune thyroid disease in population of Novosibirsk", *Clinical and experimental thyroidology,* vol. 4, no. 4, pp. 41-45, 2008 [in Russian].

22. Man-Man Lu, Qian-Ling Ye, Chen-Chen Feng et al., "Association of FAS gene polymorphisms with systemic lupus erythematosus: A case-control study and metaanalysis", *Experimental and Therapeutic Medicine,* vol. 4, pp. 497-502, 2012.