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INFLUENCE OF THE CTLA-4 (RS231775) GENE POLYMORPHISM ON THE DEGREE OF THE THYROID GLAND ENLARGEMENT IN PATIENTS OPERATED FOR NODULAR GOITER SECONDARY TO AUTOIMMUNE THYROIDITIS

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Abstract

Objectives: To analyze the influence of the CTLA-4(+49G/A)rs231775 gene polymorphism on the degree of thyroid gland enlargement in patients operated for nodular goiter secondary to autoimmune thyroiditis (NGAIT) and thyroid adenomas (TA). Also, to analyze the clinical course of the disease, depending on the genotype of this gene.
Methods: The CTLA-4 (rs231775) genes' polymorphism was studied by Real-Time Polymerase Chain Reaction in 95 patients with NGAIT, 30 patients with TA and 25 healthy individuals.
Results: It has been found that, in patients with nodular goiter secondary to autoimmune thyroiditis, hyperplasia of the thyroid gland is associated with the wild A allele of the CTLA-4 gene (AA- and AG-genotypes):

Résumé

L'influence du polymorphisme du gène CTLA-4 (RS231775) sur le degré d'hypertrophie de la glande thyroïde chez les patients opérés pour goître nodulaire secondaire à la thyroïdite auto-immune

L'objectif de l'étude a été d'analyser l'influence du polymorphisme du gène CTLA-4 (+ 49G / A) rs 231775 sur le degré d'hypertrophie de la glande thyroïde chez les patients opérés pour goître nodulaire secondaire à la thyroïdite auto-immune et aux adénomes thyroïdiens. Il analyse également le cours clinique de la maladie, selon le génotype de ce gène.
Méthodes: Le polymorphisme des gènes CTLA-4 (rs231775) a été étudié par réaction en chaîne en polymère en temps réel chez 95 patients atteints de

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IB and III levels of hyperplasia are more likely to occur in carriers of the AA genotype by 30.13% and 26.35%, and the second degree of the thyroid gland enlargement- in patients with the AG-genotype by 33.52% and 34.04%, respectively.

Conclusions: The incidence of hypertrophic form of autoimmune thyroiditis is associated with AA- and AG-genotypes of the CTLA-4 gene, characterized by a particular severity, with the rapid development of an "aggressive" proliferative process in the thyroid tissue, according to sonographic findings. The carrier of the GG genotype is associated with atrophic origin of autoimmune thyroiditis; the tissue is characterized by a high content of cells in which there are atypical or follicular changes of an unclear genesis.

Key words: nodular goiter secondary to autoimmune thyroiditis, adenoma of the thyroid gland, hyperplasia, APO-1 / FAS, CTLA-4 and BCL-2 gene polymorphisms.

Abbreviations: NGAIT – nodular goiter with autoimmune thyroiditis, AIT- autoimmune thyroiditis, TG – thyroid gland, TA – thyroid adenoma.

GNTAI, 30 patients atteints d'adénome thyroïdien (AT) et 25 personnes en bonne santé.

Résultats: Il a été constaté que chez les patients atteints de goître nodulaire secondaire à une thyroïde auto-immune, l'hyperplasie de la glande thyroïde est associée à l'allèle A sauvage du gène CTLA-4 (génotypes AA et AG): les niveaux d'hyperplasie IB et III sont plus susceptibles de se produire chez les porteurs du génotype AA de 30,13% et 26,35%, et le deuxième degré de l'élargissement de la glande thyroïde chez les patients atteints du génotype AG de 33,52% et 34,04% respectivement.

Conclusions: L'incidence de la forme hypertrophique de la thyroïdite auto-immune est associée aux génotypes AA et AG du gène CTLA-4, caractérisé par une gravité particulière avec le développement rapide d'un processus prolifératif «agressif» dans le tissu thyroïdien selon les résultats échographiques. Le porteur du génotype GG est associé à une origine atrophique de la thyroïdite auto-immune; le tissu est caractérisé par une teneur élevée en cellules dans lesquelles il y a des changements atypiques ou folliculaires d'une genèse peu claire.

Mots clés: goître nodulaire secondaire à la thyroïdite auto-immune, adénome de la glande thyroïde, hyperplasie, APO-1 / FAS, polymorphismes du gène CTLA-4 et BCL-2.

Abréviations: GNTAI – goître nodulaire avec thyroïdite auto-immune, TAI-thyroïdite auto-immune, GT – glande thyroïde, AT – adénome thyroïdien.

Introduction

The problem of autoimmune thyroiditis (AIT) is of interest, since the issues of etiology, pathogenesis, morphology, classification, diagnosis, therapy and prognosis of the disease have not yet been entirely clarified[1-3]. AIT is considered to be one of the most common diseases of the thyroid gland (TG), after iodine deficiency disorders[3]. Its prevalence among the adult population ranges from 6 to 11%[1]. It occupies a significant place in the structure of diffuse nontoxic goiter and is the most common cause of primary hypothyroidism. In Ukraine, about 60% of people with endocrine pathology suffer from different thyroid diseases, among whom about 30% are women aged over 40 years and 4.2% are children[1].

Currently, many genes are known to be involved in the development of AIT. They interact in complex ways with the environmental factors, therefore AIT is included into a group of multifactorial diseases[2-3]. A gene whose expression product (enzyme, hormone, receptor, structural or transport protein) can directly or indirectly participate in the development of pathology is called a „candidate gene". The CTLA-4 gene is considered to be one of the most likely candidate genes associated with autoimmune thyroid disease[5-9]. The cytotoxic T-lymphocyte-associated antigen (protein) –4 (CTLA-4), also known as CD152, is a member of the immunoglobulin superfamily; it is expressed on activated T lymphocytes and is a co-stimulatory molecule. CTLA-4, similar to CD28, binds to CD80 and CD86 on antigen-presenting cells (APCs) and acts as a negative regulator of T-cell activation[10-15].

Despite the fact that the CTLA-4 gene is considered to be one of the most likely candidate genes associated with AIT, the possibility of using the +49 A / G polymorphic marker to evaluate the association with AIT should be verified in each particular population.

The influence of CTLA-4 gene polymorphism on the degree of thyroid gland enlargement in patients operated for nodular goiter secondary to

autoimmune thyroiditis (NGAIT) and thyroid adenoma (TA) is poorly understood and requires further research. There have not been such studies in Ukraine.

Objective: to study the effect of the CTLA-4 (+ 49G / A) rs 231775 gene polymorphism on the degree of the thyroid gland enlargement in patients operated for nodular goiter secondary to autoimmune thyroiditis (NGAIT) and thyroid adenomas (TA) and to analyze the clinical course of the disease, depending on the genotype of the gene.

Material and methods

Between 2013-2016, on the basis of the Chernivtsi Regional Clinical Hospital, 125 women with surgical pathology of the thyroid gland were examined. 95 of them were patients with NGAIT. The age of the patients was between 23 and 72 years. The diagnosis was made clinically, in laboratory (thyroperoxidase antibodies (ATPO) – 60-250 U/mL, thyroglobulin antibody (ATTH) – 60-500 U/mL; thyroid-stimulating hormone (TSH) – 4.10 IU/L), by immunoassay and by the results of the thyroid gland sonography (shape, size, echo density, the presence of volumetric masses), fine-needle aspiration biopsy (FNAB), intraoperative express biopsy and histological examination after surgical treatment of thyroid nodules.

From all the patients, we selected a group of 30 women who, according to ultrasonography, FNAB, intraoperative biopsy and histological examination after the operation, were diagnosed with thyroid adenoma. We selected this group due to the fact that this pathology is one of the most common among nodular goiters. These patients were examined for parenchyma not affected by a nodule, contralateral lobe of the thyroid gland unaltered morphologically. These indicators served as controls. The final confirmation of the morphologically unchanged tissue was obtained after a histological examination.

All the patients underwent a surgical intervention according to generally accepted indications: large size of the goiter with compression and displacement of the neck organs (compression syndrome), airway obstruction or suspected malignant neoplasm of the thyroid gland (III, IV, V group according to the classification of the Bethesda system for reporting thyroid cytopathology) by FNAB findings. The volume of surgery was from hemithyroidectomy to thyroidectomy.

General clinical, hormonal and genetic studies were carried out in 25 practically healthy donors.

Genetic studies were performed in the Genetics Laboratory on the basis of the "N. Testemitanu" State University of Medicine and Pharmacy (Republic of Moldova). DNA was isolated from lymphocytes of venous whole blood. Venous blood was stored in test tubes, stabilized K2-EDTA. Isolation and purification of the DNA from the resulting material was carried out in accordance with the Thermo Scientific GeneJET Genomic DNA Purification kit manual. # K0721, Thermo Fisher Scientific. To standardize the conditions for the determination of polymorphisms, all samples were brought to a concentration of 2 ng/μL, by DNA dilution in nuclease-free water.

Polymerase chain reaction (PCR) was performed in real-time (RT-PCR) using Taq DNA polymerase and specific primers and probes on QuantStudio 6 Applied Biosystems (USA), which allowed to obtain amplicons, to determine their number in real time, as well as to reduce the probability of diagnostic errors. Analysis of the data was carried out using the Quant Studio Real Time Software program (Fig. 1).

The main part of the statistical analysis was conducted using the "Statistica 7.0" (SPSS) software. Nominal data are presented in the form of quantitative and percentage values. The correspondence of the distribution of genotypes to the Hardy-Weinberg equilibrium was verified using the Online Encyclopedia for Genetic Epidemiology Studies (http://www.oege.org/software/hwe-mr-calc.shtml). To compare the distribution of genotypes in the experimental and control groups, the Pearson's chi-squared test (χ^2) was used. The reliability of the differences in mean values in groups with different genotypes was determined using one-factor dispersion analysis (ANOVA). The influence of factors on the development of thyroid dysfunction was assessed using the model of binary logistic regression based on relative Relapse Ratio (RelR), Risk Relation (RR), and Odds Ratio (OR) with a 95% Confidence Interval (95% CI) based on the criterion of χ^2 (df = 1). The difference was considered to be significant at $P < 0.05$. (ANOVA). The influence of factors on the development of thyroid pathology was assessed using the model of binary logistic regression based on relative Relapse Ratio (RelR), Risk Relation (RR), and Odds Ratio (OR) with a 95% Confidence Interval (95% CI) based on the criterion of χ^2 (df = 1). The difference was considered to be reliable at $P < 0.05$.

The discrimination of genotypes of analyzed polymorphism samples of rs 231775 of the CTLA4 rs17759659 gene is shown in Fig. 1.

Results

While studying the discrimination of alleles of polymorphism rs 231775 of the CTLA4 gene (+ 49G / A), the presence of a heterozygous AG of the polymorphic variant of the gene in the patient's genome

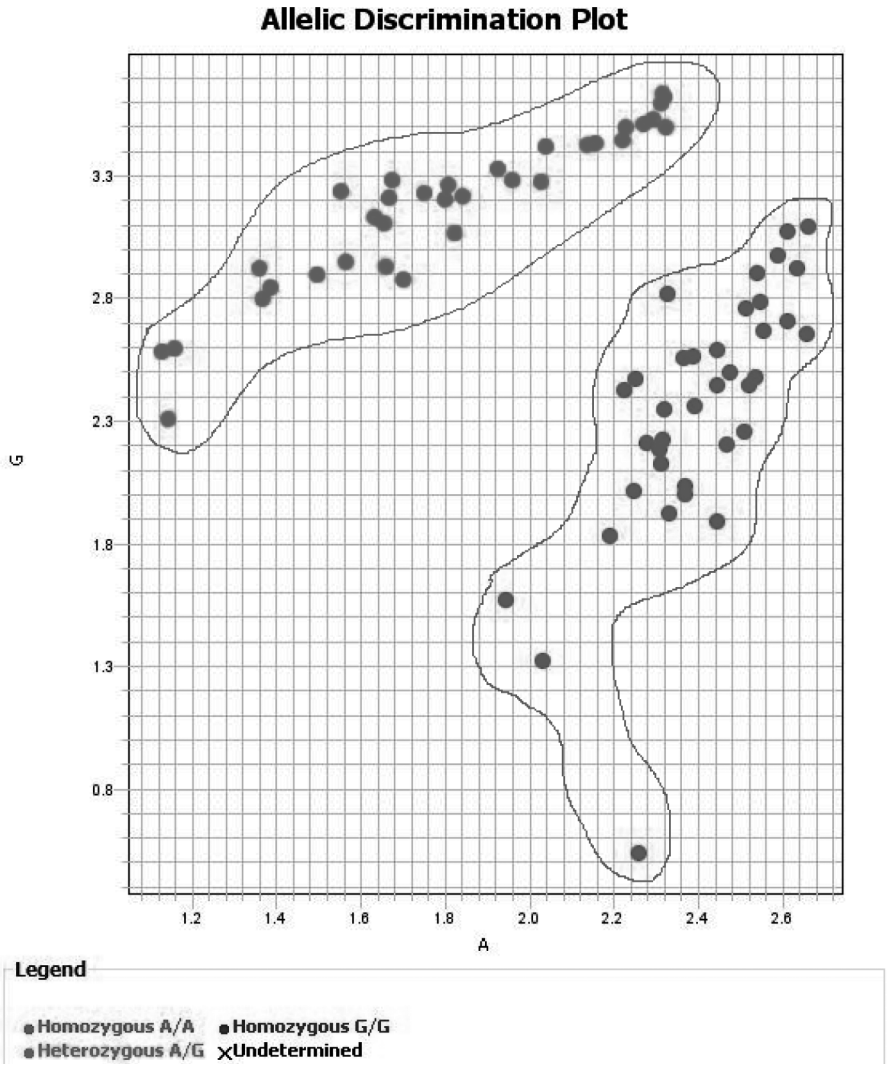

Figure 1. Discrimination of the alleles of rs 231775 *CTLA*4 (+49G/A) gene polymorphism

was noted. The gene associates with the initiation of the process of transformation and proliferation of T lymphocytes that acquired antigenic properties, increases the risk of the second degree thyroid gland hyperplasia by 2.01 times (OR = 4.69; 95% CI OR: 1.60-13.69; p = 0.004) (Table 1). Being a carrier of the main allele in the homozygous state is protective and makes the chances for the second degree thyroid hyperplasia the lowest in the surveyed population (OR = 0.46; 95% CI OR: 0.09-0.73; p = 0.009). However, the polymorphic variants of CTLA-4 (rs231775) do not associate with the risk of AIT or TA.

Thyroid hyperplasia in patients with nodular thyroid disease in general, as well as in those with NGAIT, associates with the wild A allele of the CTLA-4 gene (AA- and AG genotypes): IB and III levels of hyperplasia were more likely to occur in carriers of the AA genotype by 30, 13% and 26.35% (χ^2 = 9.26; p = 0.01), while the second stage of thyroid enlargement was by 33.52% and 34.04% in patients with the AG genotype (χ^2 = 12, 34; p = 0.002) respectively (Table 2).

Discussion

AIT belongs to the category of multifactorial pathologies with non-Mendelian pattern of inheritance. The phenotypic manifestations of genetic polymorphism largely depend on the gene pool and the living conditions of each particular population. This explains the contradiction in the data on the association of polymorphic loci (candidate genes) with the risk of development of AIT[11]. This paper studies the contribution of the +49 A / G polymorphic markers of the surface anti-gene of cytotoxic T-lymphocytes (CTLA-4) to the development of nodular pathology of the thyroid gland. The choice of genes is due to the

Table 1. Polymorphic variants of the *CTLA-4* (*rs*231775) gene as risk factors of the thyroid Pathology

CTLA-4 gene genotypes		*RelR*	*OR*	*95%CI RR*	*95%CI OR*	p
Thyroid pathology	AA	0,79	0,60	0,54-1,14	0,25-1,43	>0,05
	AG	1,38	1,75	0,79-2,39	0,72-4,25	>0,05
	GG	0,80	0,79	0,09-6,86	0,08-7,41	>0,05
Thyroid adenoma	AA	0,67	0,44	0,39-1,15	0,15-1,31	>0,05
	AG	1,67	2,67	0,92-3,03	0,89-7,98	>0,05
	GG	-	-	-	-	-
Autoimmune thyroiditis	AA	0,82	0,65	0,56-1,20	0,27-1,60	>0,05
	AG	1,29	1,53	0,73-2,27	0,62-3,81	>0,05
	GG	1,05	1,05	0,12-9,01	0,11-9,88	>0,05
First degree thyroid hyperplasia	AA	0,96	0,91	0,65-1,41	0,35-2,35	>0,05
	AG	1,08	1,14	0,59-2,0	0,43-2,99	>0,05
	GG	0,85	0,84	0,08-8,93	0,07-9,73	>0,05
Second degree thyroid hyperplasia	AA	0,46	0,25	0,25-0,83	0,09-0,73	0,009
	AG	2,01	4,69	1,15-3,51	1,60-13,69	0,004
	GG	-	-	-	-	-
Third degree thyroid hyperplasia	AA	0,90	0,78	0,56-1,45	0,26-2,36	>0,05
	AG	1,07	1,11	0,52-2,18	0,36-3,46	>0,05
	GG	1,92	2,0	0,19-19,90	0,17-23,56	>0,05

Note. *RelR* – relative risk; *OR* – Odds Ratio; *95%CI RR, OR* – confidence interval of relative risk (*RR*), of odds ratio (*OR*). TG – thyroid gland.

Table 2. Distribution of polymorphic variants of the CTLA-4 (rs231775) gene in the patients with thyroid disease considering the degree of its enlargement

The genes under study, n (%)		Control, n=25 (%)	Thyroid hyperplasia, n (%)			χ^2 p
			IB degree, n=59	II degree, n=40	III degree, n=26	
CTLA-4 (+49G/A), n (%)	AA	15 (60,0)	34 (57,63)	11 (27,50)	14 (53,85)	χ^2 =9,26 p=0,01
	AG	9 (36,0)	23 (38,98)	29 (72,50)	10 (38,46)	χ^2 =12,34 p=0,002
	GG	1 (4,0)	2 (3,39)	0	2 (7,69)	p>0,05
χ^2 p		χ^2 =36,48 p<0,001	χ^2 =40,32 p<0,001	^{2} =16,20 p<0,001	^{2} =12,92 p=0,002	-

participation of their protein products – the receptor CTLA-4 in the pathogenesis of AIT[19].

The polymorphic +49 A/G loci that we studied lie in the promoter region of the CTLA-4 gene and, according to many authors, influence the expression of the gene. Since the promoter regions are transcription factor binding sites, the regulation of gene expression is under their control. According to research results in populations of Iran, Tunisia, Germany, Canada, Norway, Japan, and others, the association of these polymorphic variants of the gene with the risk of development of autoimmune diseases and with an increase in the proportion of allele A and G by polymorphic loci +49 A/G (respectively) leads to AIT, Graves' disease, type 1 diabetes mellitus (DM1), systemic lupus erythematosus[16-21].

Probably, based on the association with the +49 A/G polymorphism that we obtained, there is an increase in the concentration of the soluble form of the CTLA-4 receptor, since single nucleotide polymorphism (SNP) of the promoter regions of the gene leads to an increase in the expression of the gene, thereby increasing the amount of the synthesized product. The polymorphism encodes the area of the gene +49 A/G and is considered to be a marker of the risk of developing various autoimmune diseases (diabetes mellitus, AIT, Graves' disease, rheumatoid arthritis, etc.) and the development of malignant neoplasms. Kotsa K. et al studied the functional role of this polymorphism and showed an increase in the proliferation of T cells in carriers of the genotype GG (Ala / Ala) compared to those of

the homozygous genotype by the normal AA allele (Thr / Thr)[12]. Many populations in the world have a high birth rate of polymorphic allele G in patients with the above-mentioned illnesses. The racial and population differences of the polymorphic variants of the CTLA-4 gene (rs231775) in the comparative aspect are presented in table 3. The frequency of the main A-allele of the CTLA-4 gene in inhabitants of Bukovina (P_A = 0.72-0.78), as well as minor G-allele (P_G = 0.22-0.28) corresponds to that on average in Caucasian populations (P_A = 0.61-0.88 and P_G = 0.12-0.39; p> 0.05), indicating a relative homogeneity of the polymorphic locus of the investigated gene. In the same time, there were significant differences compared to the individual populations of the Congoid, where the incidence of alleles and genotypes has a wide spread and discrepancy, indicating a rather high heterogeneity (P_A = 0.54-0.67 and P_G = 0.33-0.46; p <0.05, respectively). Somewhat less heterogeneity was found in populations of the Mongoloid in which the incidence of the A-allele, according to the NCBI, is reliably lower than the one found by us, and the G-allele, on the contrary, exceeds that of the inhabitants of Bukovina (P_A = 0.29-0.37 and P_G = 0.63-0.71; p <0.05, respectively)[16].

The studies conducted in Japanese[17,18], Polish[19], and Russian[20,21] populations also confirmed the presence of this association. Among all SNP variants of the TL-4 gene +49 A/G, polymorphism is most studied because it affects the function of the T-lymphocyte protein-receptor itself. This mutation is to replace adenine with guanine at position +49 exon 1, which results in the replacement of threonine with alanine in the 17 codon of the amino acid sequence of the protein. Polymorphism is associated with incomplete glycosylation of the signal peptide and an alternative splicing in the endoplasmic reticulum and thus affects the regulatory ability of the gene, which is very important in the pathogenesis of AIT.

We reflected it in our paper as well. The dominance of wild A-allele over the mutational G-allele of the CTLA-4 gene was by 2.57 times among the patients (χ^2 = 96.8; p <0.001), and by 3.54 times among healthy individuals (χ^2 = 31.36 ; p<0,001).

Analyzing the clinical findings and the treatment results, it was found that in patients with wild A allele of the gene CTLA-4 (AA- and AG-genotypes), the course of NGAIT was characterized by a particular severity, with the rapid development of an "aggressive" proliferative process in the thyroid gland itself (according to FNAB findings), by the presence of compression symptoms and the absence of the effect of conservative treatment for 1 to 3 years. It regulated the need to apply special (more active) surgical tactics in the patients with AA- and AG-genotype, with the use of early surgical treatment and the subsequent post-operative pathogenically driven therapy. In such patients, thyroidectomy was the operation of choice. After surgery, the histological conclusion confirmed the hypertrophic form of AIT secondary to nodular goiter.

In this case, the proportion of homozygous genotype GG and polymorphic allele G in the group of patients with GNAIT correlates with atrophic form of AIT and the presence of pseudonodules in one of the thyroid lobes. There was no compression syndrome in this group of patients. At the same time, the carriers of the genotype GG had the third and fourth category of risk according to the classification (The Bethesda system for reporting thyroid cytopathology) by FNAB findings, which became an indication for the operation. The operation of choice for such patients was hemithyroidectomy, with dynamic observation of patients in the remote postoperative period.

Thus, summarizing the research results, it should be noted that the incidence of genotypes AA, AG and GG polymorphism of CTLA-4 gene (rs231775) in patients with NGAIT did not differ reliably. AA, AG-genotype was identified mainly in the hypertrophic form of AIT secondary to nodular goiter. In the same time, the AG-genotype carrier can be considered as a prognostic marker for the severe NGAIT clinical course. Being a carrier of homozygous GG-genotype, in our studies, is associated with a more frequent atrophic form of AIT secondary to pseudobulbine nodules of the thyroid gland. The presence of a mutated homozygous genotype contributes to the progression of the inflammatory process

Table 3. Racial and population differences in the incidence of the CTLA-4 gene (rs231775) polymorphism

Races, populations	AA genotype	AG genotype	GG-genotype	A-allele	G-allele
Our results *(Bukovina)*	0,47-0,60	0,36-0,50	0,03-0,04	0,72-0,78	0,22-0,28
Caucasoid	0,33-0,86	0,14-0,58	0-0,14	0,61-0,88	0,12-0,39
Congoid *(African American's, sub-Saharan Africa)*	0,25-0,45	0,45-0,58	0,10-0,17	0,54-0,67	0,33-0,46
Mongoloid	0-0,36	0,08-0,49	0,39-0,92	0,29-0,37	0,63-0,71

in the thyroid tissue, with the development of a rapid, widespread lesion, confirming the sonography findings.

Conclusions

1. In patients with nodular goiter secondary to autoimmune thyroiditis, hyperplasia of the thyroid gland is associated with the wild A allele of the CTLA-4 gene (AA- and AG genotypes): IB and III levels of hyperplasia are more likely to occur in carriers of the AA genotype by 30.13% and 26.35%, and the second degree of the thyroid gland enlargement – in patients with AG-genotype by 33.52% and 34.04%, respectively.

2. The incidence of hypertrophic form of autoimmune thyroiditis is associated with AA- and AG-genotypes of the CTLA-4 gene, characterized by a particular severity, with the rapid development of an "aggressive" proliferative process in the thyroid tissue according to sonographic findings.

3. Being a carrier of the GG genotype is associated with an atrophic form of autoimmune thyroiditis; the tissue is characterized by high content of cells, in which there are atypical or follicular changes of an obscure genesis. The operation of choice for such patients should be hemithyroidectomy with dynamic observation of patients.

Conflicts of interest: The authors declared no conflict of interest

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