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# From genotype to protein: how the 308 G/A polymorphism of the TNF-α gene is associated with TNF-α protein levels in patients with Hashimoto's thyroiditis

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**Aim.** To study the 308 G/A polymorphism of the *TNF-a* gene and its influence on serum levels of TNF-a protein in patients with Hashimoto's thyroiditis (HT) among the Azerbaijan population.

**Materials and methods**. The study was conducted at the Department of Biochemistry, Azerbaijan Medical University, between 2021 and 2023. The study enrolled 170 patients diagnosed with HT, and a comparison group consisting of 65 individuals without thyroid pathology or other diseases affecting the immune system. The presence of the TNF-α-308 G/A polymorphism (SNP rs1800629) was determined by the PCR-PDRF method and TNF-α protein serum levels were measured according to the standard ELISA protocol in both groups.

**Results.** The study on the 308 G/A polymorphism of the *TNF-α* gene has revealed 49.2 % of patients exhibited the G allele and 55.1 % – the A allele. The AG genotype has been found to be most prevalent in HT patients (57.9 %) being significantly higher than in the comparison group (21.7 % of individuals; p = 0.0006, $\chi^2$ = 11.87, OR = 2.85, 95 % CI = 1.55–5.23). Furthermore, serum TNF-α levels have been shown to be significantly higher in HT patients (3.48 ± 1.32 pg/ml) as compared to the control group (2.31 ± 0.74 pg/ml) with statistical significance (p < 0.01).

**Conclusions.** The most prevalent AG genotype of the *TNF-α* gene 308 G/A polymorphism has been identified in patients diagnosed with Hashimoto's thyroiditis. This polymorphism may serve as a genetic marker, indicating a predisposition to autoimmune thyroiditis among the Azerbaijan population. Furthermore, serum TNF-α protein levels have been found to be significantly elevated in patients with Hashimoto's thyroiditis, which may be associated with the AG genotype of the 308 G/A *TNF-α* polymorphism.

**Keywords:** Hashimoto thyroiditis, SNP, TNF-α, gene polymorphism.

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## Від генотипу до білка: як поліморфізм 308 G/A гена ФНП-α пов'язаний із рівнем білка ФНП-α у пацієнтів із тиреоїдитом Хашимото

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**Мета роботи –** вивчення 308G/A поліморфізму гена ФНП-α та його впливу на рівень експресії білка ФНП-α у сироватці крові пацієнтів з тиреоїдитом Хашимото серед населення Азербайджану.

**Матеріали і методи.** Дослідження здійснено на кафедрі біохімії Азербайджанського медичного університету в період з 2021 до 2023 року. У дослідженні взяли участь 170 пацієнтів із діагнозом тиреоїдит Хашимото (ТХ), а також 65 осіб без патології щитовидної залози або інших захворювань, що впливають на імунну систему, котрі залучені до групи порівняння. У пацієнтів та обстежених із групи порівняння визначали 308 G/A поліморфізм гена ФНП-α та вміст білка ФНП-α у сироватці крові. Наявність 308 G/A поліморфізму гена ФНП-α (SNP rs1800629) визначали методом ПЛР-ПДРФ, а кількісно білок ФНП-α визначали в сироватці крові за стандартним протоколом ІФА.

**Результати.** Дослідження поліморфізму 308 G/A гена ФНП-α показало: хоча 49,2 % пацієнтів мали алель G, а 55,1 % – алель A, наявність генотипу AG у пацієнтів з ТХ найбільш поширена – 57,9 %. Цей показник значно вищий щодо групи порівняння, де генотип AG встановлено у 21,7 % обстежених (p = 0,0006, $\chi^2$ = 11,87, OR = 2,85, 95 % CI = 1,55–5,23). Крім того, виявлено, що вміст ФНП-α у сироватці крові значно вищий у пацієнтів із ТХ (3,48 ± 1,32 пг/мл) порівняно з обстеженими з контрольної групи (2,31 ± 0,74 пг/мл). Ці відмінності статистично значущі (p < 0,01).

**Висновки.** У пацієнтів із діагнозом ТХ виявлено найбільш поширений генотип AG поліморфізму 308 G/A гена ФНП-α. Цей поліморфізм можна використовувати як генетичний маркер, що вказує на схильність до автоімунного тиреоїдиту в азербайджанській популяції. Крім того, у пацієнтів із ТХ встановлено значне підвищення рівня білка ФНП-α у сироватці крові, що може бути пов'язано з генотипом AG поліморфізму 308 G/A гена ФНП-α.

**Ключові слова:** тиреоїдит Хашимото, SNP, ФНП-α, поліморфізм генів.

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The necessity to study genetic factors emerged due to their direct correlation with biochemical processes, given that genes alone do not execute biological functions. As is well recognized, they function as a matrix for the synthesis of proteins, which are pivotal molecules in ensuring cellular processes. Single nucleotide polymorphism (SNP) has been shown to result in alterations to the protein structure and function, which in turn exerts an influence on the regulation of metabolic pathways, enzyme activity, and molecule interactions [1]. The study on candidate genes aims to identify the mechanisms underlying abnormalities of biochemical processes associated with Hashimoto's thyroiditis (HT) [2].

Following a comprehensive review of the available literature and considering methodological affordability, the study was focused on TNF-α gene polymorphism, a candidate gene for autoimmune disorders. Gene product – TNF-α is a potent proinflammatory and immunoregulatory cytokine that is synthesized by cells such as macrophages, monocytes, neutrophils, T cells, and NK cells [3].

TNF-α protein has a wide range of biological activities with diverse functions in humans, including induction of apoptosis, lipid metabolism and coagulation, regulation of lymphocyte proliferation, increasing chemokine levels, activation of macrophages and neutrophils [4]. TNF-α affects the T and B cell activity, playing an important role in the adaptive immune response. It promotes antibody production and increases the activity of cytotoxic T cells [5,6]. TNF-α protein stimulates the production of various proteolytic enzymes, including metalloproteinases, in particular MMP-3 [7]. The main biological function of TNF-α is to induce inflammation through the gene transcription regulation, which leads to the expression of a large number of genes encoding proteins participating in the above processes [8].

Studies have shown a major impact of the 308 G/A polymorphism of the *TNF-α* gene (rs1800629) on transcriptional variation resulting in plasma TNF-α level changes [9].

Therefore, the following mechanisms can lead to the development of autoimmune reactions:

– stimulation of thyrocytes to increased expression of class II MHC molecules, which turns thyrocytes into 'targets' for attack by their own organism [10];

– production of other pro-inflammatory proteins that increase the inflammatory process in thyroid tissue, which eventually leads to tissue destruction [11];

– initiation of apoptosis mainly through activation of Fas/FasL signalling pathways [12];

– production of reactive oxygen species (ROS) damaging thyroid cells, which triggers autoimmune reactions (the immune system identifies damaged cells as foreign) [13];

– disruption of the regulatory T cell (Treg) functions, which leads to an uncontrolled immune response against thyroid tissue [14].

This study is focused on the determination of possible allelic and genotype variants of the *TNF-α* 308 G/A polymorphism in patients among the Azerbaijan population with HT, that has not been attempted before.

## Aim

The aim of this study is to examine the *TNF-α* 308 G/A polymorphism and its influence on serum TNF-α protein levels in patients with Hashimoto's thyroiditis in the Azerbaijan population.

## Materials and methods

The work was carried out at the Biochemistry Department of Azerbaijan Medical University in 2021–2023. Blood from 170 patients with a primary diagnosis of HT was examined. The comparison group consisted of 65 individuals without thyroid pathology or other autoimmune diseases. The groups were comparable by sex and age ($p = 0.6155$ and $p = 0.3093$, respectively). The group with HT consisted of 64 (37.6 %) men and 106 (62.4 %) women with a mean age of 42.5 years (Me = 43.0; Q1 = 33.0, Q3 = 52.0).

The comparison group consisted of 26 (40 %) men and 39 (60 %) women with a mean age of 42.8 years (Me = 43.0; Q1 = 35.0, Q3 = 54.0).

The diagnosis of HT was made by clinicians based on the results of clinical and hormonal examinations, determination of antibodies to thyroid peroxidase (TPO) and thyroid ultrasonography. Inclusion criteria were as follows: primary diagnosed HT, absence of concomitant allergic or other autoimmune disorders, no severe somatic diseases.

Criteria for exclusion: comorbid pathology associated with HT by common pathogenetic mechanisms resulted from aggravated autoimmune processes; pregnancy or lactation; acute or chronic inflammatory processes.

Blood samples were collected in the Endocrinology Department of the Scientific and Surgical Centre named after Academician M. A. Topchubashev. In both groups (patient group and comparison group), the presence of *TNF-α* 308 G/A polymorphism and serum TNF-α protein levels were determined. The presence of the 308 G/A polymorphism of the TNF-α gene (SNP rs1800629) was detected by PCR-PDRF with NcoI restriction enzymes (New England Biolabs Inc.) being utilized in the process. The following primers were used for the necessary DNA fragment amplification: 5'-AGGCAATAGGTTTTTGAGGGGGCCAT-3' – forward primer; 5'-TCCTCCTCCCTGCTCCGCTCCGATTCCG-3' – reverse primer. PCR was performed using a set of primers targeting the corresponding genomic regions followed by restriction enzyme analysis for result interpretation. The amplification was carried out on a CFX96 amplifier (Bio-Rad) with subsequent visualization and evaluation of results in the Bio-Rad CFX-96 software.

A kit for Tumor Necrosis Factor Alpha (TNF-α) (Cloud-Clone Corp. (CCC, Wuhan)) to quantify serum TNF-α was used via sandwich enzyme immunoassay. The methodology followed the standard ELISA protocol with an automatic analyzer "EL 808 Bio-Tek Instruments, Inc." (Diagnostic Products Corporation, USA).

The subsequent statistical analysis was conducted using Statistica 12 software. Genotype frequencies were assessed regarding the Hardy–Weinberg equilibrium using the chi-squared ($\chi^2$) criterion. Finally, the relative risk of the disease development was calculated using the odds ratio (OR). It should be noted that for all statistical calculations, a p-value of less than 0.05 was considered to be statistically significant.

The results for quantitative variables were presented as a median (Me) and interquartile range (Q1; Q3). The

**Table 1.** Distribution of allele and genotype frequencies of the *TNF-α* – 308 A/G gene polymorphism in HT patients and controls

| Alleles and genotypes | HT, n = 170 | Control group, n = 65 | p | $\chi^2$ | OR | 95 % CI |
|---|---|---|---|---|---|---|
| AA, n (%) | 41 (24.1) | 42 (64.6) | <0.0001 | 33.76 | 0.174 | 0.094–0.323 |
| AG, n (%) | 95 (57.9) | 20 (21.7) | 0.0006 | 11.87 | 2.85 | 1.550–5.230 |
| GG, n (%) | 34 (20.0) | 3 (4.6) | 0.0038 | 8.39 | 5.176 | 1.200–17.460 |
| A, n (%) | 97 (55.1) | 48 (43.8) | 0.0179 | 5.61 | 0.471 | 0.250–0.885 |
| G, n (%) | 73 (49.2) | 17 (26.1) | 0.0179 | 5.61 | 2.125 | 1.131–3.994 |

non-parametric Mann–Whitney test was used to assess differences in TNF-α levels between the patient group and the control group, as the distribution of TNF-α concentration data did not follow a normal distribution (verified using the Shapiro–Wilk test) in both groups.

All the participants signed written informed consent to participate in the study. The study was approved by the Ethics Committee of Azerbaijan Medical University (Ref. no: AMU/IEC/№12/07.02.2020).

## Results

Significant differences have been found in distribution of genotype and allele frequencies of polymorphic 308 A/G of the *TNF-α* gene between the studied groups *(Table 1)*. The analysis has shown the highest AG genotype prevalence in HT patients as compared to other genotypes and alleles, although the G allele has been detected in 49.2 % of patients and the A allele – in 55.1 % of patients. So, the AG genotype of the 308 A/G polymorphism has been detected in 57.9 % of patients being significantly more frequent in comparison to that in the control group. In the group of healthy individuals, the AG genotype has been detected in 21.7 % of participants ($p = 0.0006$, $\chi^2 = 11.87$, OR = 2.85, 95 % CI = 1.55–5.23).

Measurements of serum TNF-α have revealed significantly higher its levels (3.48 ± 1.32 pg/ml) in HT patients than those in controls (2.31 ± 0.74 pg/ml), $p < 0.01$. Although we have not statistically compared TNF-α levels between the different genotypes in the patient group, the individual data analysis has shown relatively higher TNF-α levels in patients with the AG genotype as compared with other genotypes.

## Discussion

So, our study has documented a statistically significant AG genotype frequency of the *TNF-α* – 308 A/G gene polymorphism in HT patients as compared to the group of healthy individuals in the Azerbaijan population. On the other hand, *TNF-α* encoded protein has been significantly elevated in the patient group as compared to the controls. Certainly, this suggests a role of the AG genotype in the pathogenesis of thyroiditis with pathology induced by increased serum proinflammatory protein TNF-α levels.

Controversial data on the *TNF-α* – 308 A/G polymorphic variant effects on the TNF-α expression and the protein levels can be found in the literature. For example, according to the results of a study by G. Zazeckyte, G. Gedvilaite et al. on the *TNF-α* gene different polymorphism (*TNF*-863A/C (rs1800630), *TNF*-308A/G (rs1800629), *TNF*-238A/G (rs361525)) effects on the corresponding protein level, the *TNF*-308A/G polymorphism has been found to be associated with decreased serum concentrations of TNF-α protein [15].

Other authors have concluded about an association between the *TNF-α*-308G/A SNP and increased TNF-α levels in addition to a number of infectious and metabolic diseases [16,17].

Some authors have suggested that discrepancies in results and disagreements might be partly due either to differences in ethnicity or sampling, or to a variety of other molecules interacting with the *TNF-α* promoter region and affecting the expression [18,19,20].

The matter is that the TNF-α protein, which is the corresponding gene product, exists in two forms. Its soluble form is produced from the transmembrane form and involved in autoimmune disorder induction, while the transmembrane form exerts paracrine functions. Meanwhile, extracellular segment hydrolysis to generate the soluble form depends on many factors, including activation of extracellular matrix enzymes. It is the soluble form that causes proinflammatory effects throughout the body via systemic circulation, inducing the synthesis of cytokines [21].

## Conclusions

1. In patients diagnosed with Hashimoto's thyroiditis, the AG genotype of the *TNF-α*-308 G/A polymorphism is the most prevalent (57.9 %), suggesting its potential as a genetic marker for predisposition to autoimmune thyroiditis within the Azerbaijan population.

2. Serum TNF-α levels are significantly elevated in patients with Hashimoto's thyroiditis.

3. The AG genotype of the *TNF-α*-308 G/A polymorphism is associated with increased TNF-α protein levels in patients with Hashimoto's thyroiditis in the Azerbaijan population.

**Perspectives of subsequent scientific research** include the study on *TNF-α* polymorphism in large samples and serum TNF-α protein levels in individuals with different allelic and genotype variations, that will be useful both diagnostically and prognostically in the dynamics of autoimmune thyroiditis.

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