Are single nucleotide polymorphisms in the IL-2 gene biomarkers for Hashimoto's thyroiditis?

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Abstract

Background and aims. *Hashimoto's thyroiditis* (HT) is an autoimmune disorder that can lead to hypothyroidism. The pathophysiology of HT involves the production of antithyroid antibodies that attack the thyroid tissue, causing inflammation and progressive fibrosis. Recent studies demonstrated a strong correlation between Interleukin-2 (IL-2) levels and the development of autoimmune diseases, suggesting that this cytokine may play a crucial role in the pathogenesis of HT.

Methods. In this study, we determined the presence of the point mutation +114T/G in the *IL-2* gene in patients with HT compared with a control group, and also the serum level of anti-thyroid peroxidase (TPOAbs) and anti-thyroglobulin (TgAbs) antibodies in HT patients with vs. without the mutation. The sequences of the *IL-2* gene obtained from subjects were determined by the Sanger sequencing method.

Results. Our study did not reveal that the +114T/G polymorphism of the *IL-2* gene is a susceptibility or protective factor for HT. No significant correlations were observed between the reference genotype, hetero- and homozygous +114T/G polymorphism and TPOAbs, respectively TgAbs serum levels in HT patients.

Conclusions. Further studies of more cases are needed to identify more polymorphisms in the *IL-2* gene and study their correlations with HT.

Keywords: *IL-2* gene, Hashimoto's thyroiditis, antibodies, Sanger sequencing method, thyroid autoimmune pathology

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Introduction

Hashimoto's thyroiditis (HT) is an autoimmune disorder that can lead to hypothyroidism [1]. The annual incidence of HT worldwide is estimated to be around *0.3-1.5 cases per 1,000 persons* [1]. The pathophysiology of HT involves the production of antithyroid antibodies that attack the thyroid tissue, causing inflammation and progressive fibrosis [1-3]. The diagnosis can be challenging and consequently, the condition is sometimes not diagnosed until late in the disease process. Even though the immunological mechanisms underlying this disease have been intensively studied in recent years, many aspects of its pathogenesis remain unclear [1].

Anti-thyroid peroxidase (TPOAbs) and anti-thyroglobulin (TgAbs) autoantibodies have elevated blood levels in HT [1-3]. Thyroid peroxidase is a key enzyme involved in thyroid *hormone synthesis* [4]. Antibodies are produced mainly by lymphocytes infiltrating the thyroid gland. TPOAbs levels are associated with degree of infiltration by lymphocytes, which may sensitize and trigger the synthesis of autoantibodies. TPOAbs, in contrast to TgAbs, are able to promote cellular cytotoxicity via complement activation pathways [4-6].

One of the most important factor involved in the immune response is Interleukin-2 (IL-2), a lymphokine product of activated T-lymphocytes with

immunomodulating and antitumor activity [7]. IL-2 is responsable for T-lymphocytes activation and proliferation [2,3]. Recent studies demonstrated a strong correlation between IL-2 levels and the development of autoimmune diseases, suggesting that this cytokine may play a crucial role in the pathogenesis of HT [1-3]. IL-2 can promote the expansion and differentiation of different immune cell subsets dose-dependently. At high doses, IL-2 can promote the differentiation and expansion of effector and memory T cells, whereas at low doses, IL-2 can promote the differentiation, survival and function of regulatory T cells, a CD4+ T cell subset that is essential for the maintenance of immune homeostasis [2,3,8,9].

IL-2-producing and IL-2-reactive cells appear to be present locally in autoimmune thyroiditis, although IL-2 is not the only lymphokine secreted by intrathyroidal lymphocytes [10]. Within the inflammatory cells and thyroid follicular cells, the presence of multiple cytokines, such as IL-1, IL-2, IL-6, IL-8, IL-10, IL-17 and tumor necrosis factor α (TNF-α), has been demonstrated [11-13]. In cell cultures, IL-1 and IL-6 increase the proliferation of the thyroid follicular cells, but they also have inhibitory effects on thyrocytes during stimulation of these cells by thyroid-stimulating hormone (TSH) [14].

The rapid development of molecular technologies and techniques has enabled the accumulation of a significant amount of data on IL-2 and its role in autoimmune diseases. It appears that the regulation of the IL-2 level or its receptors may have a therapeutic potential in the treatment of autoimmune diseases, including Hashimoto's disease [15]. The serum concentration of the soluble IL-2 receptor (sIL-2R) was significantly increased in patients with Basedow-Grave's disease (BGD) compared to controls [16]. On the same note, the administration of L-thyroxine in patients with HT induced a reduction in its concentration [16]. No correlation was observed between the serum levels of sIL-2R and those of TgAbs and TPOAbs in BGD and HT patients [16].

IL-2 immunotherapy administered to cancer patients has been observed to induce thyroid dysfunction [17-20]. A study performed in the USA and published in 1992 concluded that IL-2 administration as antineoplastic therapy was associated with the development of transient, subacute, painless thyroiditis without detectable antithyroidal antibodies [18]. Another study achieved in Italy published in 2002 showed that prolonged administration of high doses of recombinant IL-2 in acute myelogenous leukemia patients resulted in severe cases of hypothyroidism, which enforced the cessation of IL-2 administration and thyroid hormone replacement therapy [20].

IL-2 and its receptor (IL-2R) play a central role in the regulation of the immune response by promoting the growth and activation of T lymphocytes [16,21]. In the context of autoimmune diseases, including thyroid conditions, dysfunctions in the IL-2/IL-2R pathway can influence the development and progression of the disease by modulating the immune response. Studies in this field could explore how variations in IL-2 production or sensitivity, or mutations in its receptor gene, may contribute to the pathogenesis of thyroid conditions, such as HT or BGD, by affecting the balance between different subpopulations of T lymphocytes, including regulatory and effector cells [16,21].

This study investigated the relationship between the presence of the point mutation +114T/G in the *IL-2* gene in patients with Hashimoto's disease compared with the control group, and the serum level of TPOAbs and TgAbs antibodies in HT patients with vs. without this mutation, respectively.

Methods

Subjects

This study is a case-control type, carried out in the period 2019-2021, in the North West of Transylvania (Romania). The control group included a number of 40 subjects from the Integrated Outpatient Department of the Cluj-Napoca County Clinical Hospital of Infectious Diseases without thyroid pathology or other comorbidities. The case group included 40 patients with HT from the Cluj-Napoca County Clinical Hospital of Infectious Diseases. The matching between the groups was done according to two criteria: the gender of the study participants (31 women and 9 men in each group) and the age of the study participants (in the control group the average age was 48.33 +/- 11.88 years, in case group average age 47.35 +/- 11.18 years, the age range being 20-69 years in both groups). There were no kinship relationships between the study participants.

Study approval for the collection and analysis of biological samples from the subjects

The present study had the approval of the ethics commissions of Iuliu Haţieganu University of Medicine and Pharmacy Cluj-Napoca and Clinical Hospital of Infectious Diseases Cluj-Napoca (269 from July 30, 2019, and respectively 7692 from May 13, 2021). The following principles were respected: the principles of the Helsinki declaration of 1975, the European Convention of Oviedo of April 4, 1997 for the protection of human rights and the dignity of the human being in relation to the applications of biology and medicine. Prior to their inclusion in the study, all participants enrolled signed the informed consent for genetic and molecular biology research studies, and all obtained results were kept in an anonymous manner.

Laboratory investigations

Thyroid markers analysis

The paraclinical tests, as an inclusion criterion in HT or control group, were performed from the serum of each person enrolled in this study, and targeted the following positive markers: free thyroxine (FT4) < 1.12 IU/mL, thyroid stimulating hormone (TSH) > 5.6 IU/mL, TgAbs

> 10 IU/mL, TPOAbs > 9 IU/mL, and were determined by the chemiluminescent immunoassay method, using the Unicel DXI 800 Analyzer (Beckman Coulter, Brea, CA, US). Regarding immunological tests, blood samples were collected in a sterile 9 mL vacutainer without anticoagulant and centrifuged for 10 minutes at 1,800x *g* to obtain the serum.

Molecular and genetic analyses

DNA extraction

DNA was extracted from each subject in a sterile 1.5 mL tube using the QIAGEN DNA Kit (Qiagen, Hilden, Germany) based on special filter columns. 200 µL of whole blood were added into the microcentrifuge tube, after prior pipetting 20 µL of proteinase K, and 200 µL of Buffer AL were dispensed followed by vortexing for 15 seconds. The mixture resulted for each sample was incubated at 56°C for 10 minutes and then was briefly centrifuged. 200 µL of 99.99% ethanol was added, then the 1.5 mL tube was vortexed for 15 seconds and after that then briefly centrifuged. The mixture was applied to the QIAamp spin column in a 2 mL collection tube and after closing the cap was centrifuged at 6,000x *g* for 1 minut. The QIAamp spin column was placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. After adding of 500 µL Buffer AW1 into QIAamp spin column, another centrifugation was performed at 6,000x *g* for 1 minute. The QIAamp spin column was placed in another clean 2 mL collection tube, 500 µL of Buffer AW2 was added and a full speed centrifugation at 20,000x *g* was performed for 3 minutes. The QIAamp spin column was placed in a new clean 2 mL collection tube, and the tube with the collected filtrate has been removed. After a new full speed centrifugation for 1 minute, the QIAamp spin column was placed in a clean 1.5 mL microcentrifuge tube and the collection tube with the filtrate was discarded again. 200 µL of Buffer AE was added into QIAamp spin column, incubated at room temperature for 1 minute and then centrifuged at 6,000x *g* for 1 minute. In parallel, the purity of the extracted DNA was analyzed using the spectronanophotometer (NanoPhotometer P300, Implen GmbH, Munich, Germany). In general, a 200 µL sample of whole human blood typically yields 6 µg of DNA in 200 µL ultrapure water (30 ng/µL), with an A260/A280 ratio between 1.7-1.9. On average, a DNA concentration of 22 ng/µL +/- 1.2 was obtained, with a purity at 260/280 nm of 1.7 +/- 0.17. All DNA samples were stored at -20 °C until genotyping.

Genotyping

To perform the Polymerase Chain Reaction (PCR) amplification, the following reagents were mixed to a final volume of 25 µL: in 12.5 µL Mastermix MyTaq™Red Mix (Bioline, London, UK), 9.5 µL of water-free of DNA/RNA-ase, 1 µL of each type of primer (forward and reverse primer), and finally 1 µL of genomic DNA were added.

The fragment amplified by the PCR method had a length of 262 nucleotides. The following primers 5′-ATGTACAGGATGCAACTCCT-3′ and 5′-TGGTGAGTTTGGGATTCTTG-3′ (Generi Biotech, Hradec Králové, Czech Republic) were used, made according to available data [15] and PCR amplification was performed in a Biometra-TProfessional Basic Thermocycler (Analytik Jena GmbH, Jena, Germany). The initiation of the PCR reaction was carried out by activating the enzyme at 94 °C for 5 minutes, followed by 35 consecutive cycles that included DNA denaturation at 94 °C for 30 seconds, primer alignment at 54.5 °C for 30 seconds and extension at 72 °C for 30 seconds, and final extension at 72 °C for 10 minutes. All PCR amplicons were subjected to purification with the ISOLATE II PCR Gel Kit (Bioline, London, UK).

Sanger sequencing

This determination was performed by Humanizing Genomics Macrogen Europe Company (Amsterdam, The Netherlands). The obtained sequences were edited and analyzed using free open-source bioinformatics software kits such as UGENE version 40.1 (Unipro, Novosibirsk, Russia).

Statistical analysis

For statistical we used Microsoft Excel (2019) and R Commander, R version 4.0.5, freely available online [R Foundation for Statistical Computing, Vienna, Austria]. To compare the frequencies for categorical data, independent groups, and chi square test. Shapiro-Wilk tests, Kolmogorov-Smirnov tests, skewness, and kurtosis were used to assess quantitative data distribution. For the quantitative variables, the mean and standard deviation (in case of normal distribution) or median and ranges (for non-normal distribution) were calculated. Mann-Whitney and Kruskal-Wallis tests for the independent groups were used when we compared two and more than two groups, respectively. The results with a *p*-value less than 0.05 were considered statistically significant. Our hypothesis was that TgAbs and TPOAbs values were significantly different depending on the genotype.

Results

Clinical and paraclinical (baseline free T4, baseline TSH, TPOAbs, TgAbs, Anti TSH) data of our HT case and control groups are presented in table I.

The DNA samples amplified by PCR collected from patients with HT and control subjects examined for genotyping by Sanger DNA sequencing revealed the reference sequence of the *IL-2* gene, as well as +114 T/G heterozygous and homozygous polymorphisms (Figure 1).

Analyzing the *IL-2* gene by the Sanger sequencing method, we observed the presence of the normal allele in 23 patients with HT compared to 21 subjects in the control group, the heterozygous +114 T/G polymorphism in 11 patients with HT in contrast to 9 subjects in the control group, respectively the +114 T/G homozygous polymorphism in 6 patients with HT versus 10 control subjects. We were unable to demonstrate that the +114 T/G polymorphism of the *IL-2* gene is a susceptibility or protective factor for HT (Table II).

Table I. Clinical and paraclinical data of patients with HT and control group.

Variable	HT[1] Patients (n^2 = 40)	Control Subjects (n^2 = 40)
Female gender	31 (77.5)	31 (77.5)
Male gender	9 (22.5)	9 (22.5)
Age	47.35 +/- 11.18	48.33 +/- 11.88
Baseline FT4[3] (ng/dL)	1.57±3.06	0.80 ± 0.13
Baseline TSH[4] (mU/mL)	9.4 ± 27.9	2.65 ± 1.19
TPOAbs[5] (mU/mL)	183.64± 225.40	3.94 ± 2.59
TgAbs[6] (mU/mL)	112.85± 209.51	2.25 ± 0.71
Anti TSH (positive)	0	0

[1]HT – Hashimoto's thyroiditis, 2n – number of participants, 3FT4 – free thyroxine (reference values<1.12 IU/mL for HT group), [4]TSH – thyroid stimulating hormone (reference values>5.6 IU/mL for HT group), [5]TPOAbs – anti-thyroid peroxidase antibodies (reference values>9 IU/mL for HT group), [6]TgAbs – anti-thyroglobulin antibodies (reference values>10 IU/mL for HT group)

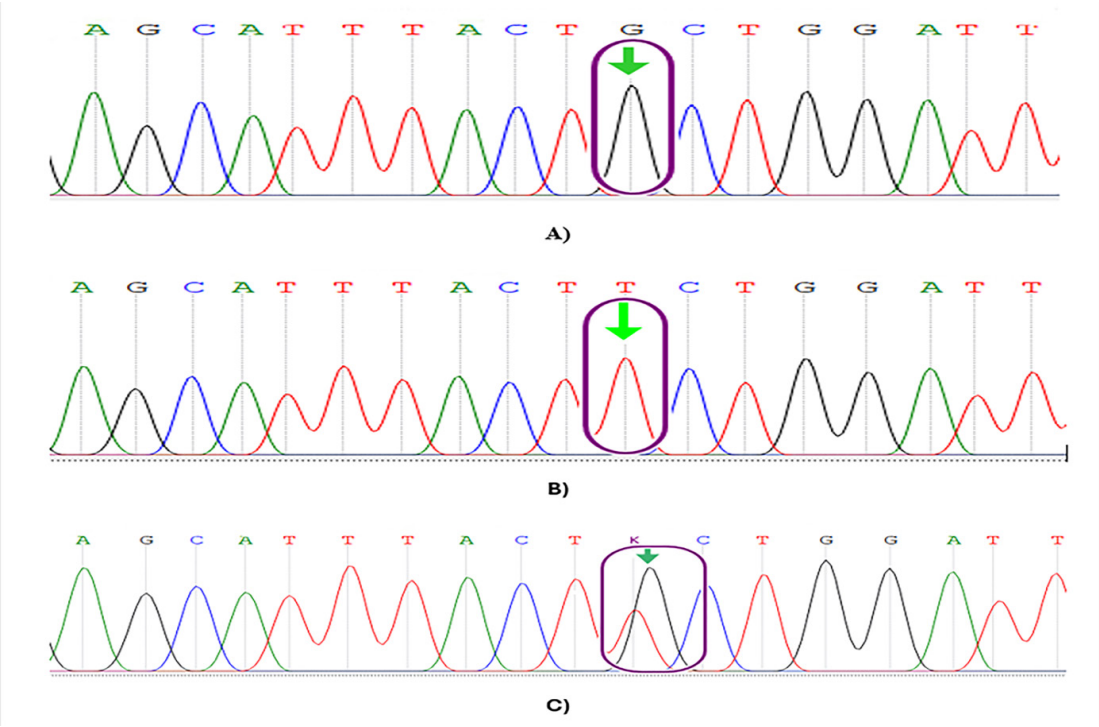

Figure 1. Chromatograms derived from Sanger sequencing of the *IL-2* gene in individuals diagnosed with Hashimoto's thyroiditis and the control group. A) The normal gene sequence with present G nucleotide at position 114. B) The variant gene sequence demonstrating T nucleotide at position 114. C) The heterozygous Single Nucleotide Polymorphism sequence K, with both G and T nucleotides at position 114.

Table II. Distribution of the +114 T/G *IL-2* gene polymorphism in the group of patients with Hashimoto's thyroiditis (HT), compared to the control group.

Allelic variant	HT group n = 40 (%)	Control group n = 40 (%)	OR	95% CI	p
Genotype					
GG	23 (57.5)	21 (47.5)	1.22	0.5–2.96	0.65
GT	11 (27.5)	9 (45)	1.3	0.47–3.6	0.6
TT	6 (15)	10 (7.5)	0.52	0.17–1.63	0.26
GT/TT	17 (42.5)	19(52.5)	0.82	0.33-1.97	0.65
Allele frequency					
G	57 (71.25)	51(63.75)	1.4	0.73–2.74	0.31
T	23 (28.75)	29 (36.25)	0.71	0.37–1.38	0.31

Table III. TPOAbs and TgAbs concentrations depending on genotype GG and GT/TT of *IL-2* gene for patients with HT disease (n=40).

Antibodies	HT group		p
	GG (23 cases)	GT/TT (17 cases)	
TPOAbs[1]	108.1 (324.7-189,5)	119.2 (62.1-411.2)	0.62
TgAbs[2]	14 (3.16-175.72)	10.6 (0.91-31,5)	0.13

[1]TPOAbs – anti-thyroid peroxidase antibodies, [2]TgAbs – anti-thyroglobulin antibodies

Also, we could not establish any statistically significant correlation between the blood concentrations of TPOAbs and TgAbs in HT cases, on the one hand, and the reference genotype, respectively that with the hetero- and homozygous +114 T/G polymorphism, on the other hand (Table III).

Discussion

Because we could not prove that the +114 T/G polymorphism of the *IL-2* gene is a susceptibility or protective factor for HT, our study suggests that this polymorphic variant cannot be used as a predictive marker for Hashimoto's thyroiditis.

We have chosen to analyze the polymorphism +114 T/G based on a study published in 2014 which investigated the association of IL-2 polymorphisms and IL-2 serum levels on Chinese Zhuang population and proved the significant influence of this polymorphism on the IL-2 serum level [22].

We used Sanger sequencing because it is the gold standard for determining polymorphisms through point mutations. Also, we chose this technique for sequencing over next-generation sequencing (NGS) due to its high accuracy, ease of use, low costs, and high precision in point mutation polymorphism analysis. Another important reason consists of the possibility to discover additional point mutations in comparison to classical PCR-based methods.

The limitations of our study are the relatively small sample size and the analysis of a single polymorphism. Given the general lack of research on the *IL-2* gene in relation to HT, there is a need for extensive studies on the correlations between polymorphisms of the *IL-2* gene and other cytokines with autoimmune thyroid diseases.

Another polymorphism in the *IL-2* gene has been investigated in relation to HT by a study conducted in Iraq published in 2024, which did not prove the influence of the *rs2069762* (-330 T/G) polymorphism in the *IL-2* gene as a susceptibility or protective factor against HT [23]. However, this research revealed statistically significant higher IL-2 serum concentrations in HT patients compared to healthy subjects, suggesting the potential for an augmented immune response that could contribute to the pathogenesis of HT [23]. Based on this evidence reported by the mentioned study, we did not determine serum concentrations of IL-2 in cases with HT compared to controls. On the other hand, it did not detect statistically significant correlations between *IL-2* gene genotypes and IL-2 serum levels in HT patients [23]. Like our study, the Iraqi one did not detect statistically significant correlations between IL-2 genotypes and the risk of developing HT [23].

Cytokines in many cases exhibit a pleiotropic action and often may function as both an immunostimulating and an immunosuppressive agent, depending on the type and location of the target. It has been reported that polymorphisms in the *IL-2* gene are associated with various cancers [24-26] and other autoimmune disease [27]. A study carried out in Poland published in 2013 highlights the association of the *rs6822844* polymorphism of the *IL-2* gene with type 1 diabetes and the influence on the serum level of IL-2 [27]. In contrast, there are very few studies on the effect of polymorphisms in the *IL-2* gene on the development of HT. Another study performed in Poland and published in 1998 investigated the relationship between serum cytokine levels and thyroid activation in BGD [28]. Mainly studied were IL-2, human tumor necrosis factor (TNFh), IL-6, and their soluble receptors (sIL-2R, sTNFhR, sIL-6R), as well as IL-10 [28]. In a small sample of eight BGD cases and nine healthy subjects, it was observed that serum IL-2 concentrations were not different in those with the disease compared to controls, but the sIL-2R level in BGD patients tended to be higher compared to controls, but without statistical significance [28]. A study conducted in China published in 2015 highlights the homozygous GG polymorphism in the -330 locus of the *IL-2* promoter gene statistically significantly associated with BGD, which supports the hypothesis of the involvement of *IL-2* genetic polymorphisms in thyroid autoimmune diseases [29].

Conclusions

Our study suggests that the polymorphic variant of the *IL-2* gene, characterized by the presence of the +114T/G point mutation, cannot be used as a predictive marker for Hashimoto's thyroiditis. No significant correlations were observed between the reference genotype, hetero- and homozygous +114T/G polymorphism and TPOAbs, respectively TgAbs serum levels in HT patients. Further studies of more cases are needed to identify more polymorphisms in the *IL-2* gene and study their correlations with Hashimoto's disease.

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