# Genetic polymorphism of *CTLA4* with functional thyroid tests in hypothyroid disease patients

**Shahad Tareq Rradeef[1*]; Elham A. Mahdi[2]**

**Dept. of chemistry, Faculty of Education for Girls, , University of Kufa**

***Corresponded author email: shahadshahad19881988@gmail.com**

## Abstract

**Objective**: To recognize the role of Cytotoxic T-Lymphocyte Antigen 4 *CTLA4* gene polymorphism **(rs231775A/G)** in Hashimoto's thyroiditis (HT) patients and a study its correlation with other clinical data.

## Design and Methods

Case-control research was conducted on 200 females, with 100 patients with Hashimoto's thyroiditis aged 18-68 years and 100 age and gender-matched healthy volunteers. Patients were drawn from Al-Sadder Medical City in Al-Najaf Governorate. All participants underwent to medical examinations to ensure they were suffering from Hashimoto's thyroiditis. The Real-time-PCR method was used to analyze the genotyping of the *CTLA4* gene, and the levels of T3, T4, TSH, Anti-TPO, and Anti-TG were assessed using an ELISA kit in all study groups.

## Results

The current study recorded that allelic and genotypic distributions of CTLA4 polymorphism at position (rs231775A/G) were not significantly different between control subjects and HT patients for the GA genotype (OR=1.50, %95 CI: (0.25-9.20), P=0.661), for the AG genotype. While for GG genotype (OR=1.7, %95 CI: (0.22-13.41), P=0.61). The same result was obtained when comparing healthy control and HT patients in the dominant, recessive, and additive models. Additionally,

a non-significant association of the *CTLA4* polymorphism (rs231775A/G) in Codominant and dominant models with TPO, Anti-TG, and other biochemical features, Age, BMI, T3, T4, TSH, fT3, fT4.

In conclusion, *CTLA4* polymorphism(rs231775A/G) was not associated with HT disease susceptibility in the present study population, With the limitation of the present small sample size, additionally, the present research suggests that CTLA4 maybe have an endogenous immunoregulatory effect, nonrelated to any specific role as a viral receptor.

**Key Words:** *CTLA4*, Hashimoto's thyroiditis, autoimmune thyroid disease.

## Introduction

Hashimoto's thyroiditis (HT) is an autoimmune disease, that can cause hypothyroidism in which the immune system turns against thyroid glands, in another meaning, the immune system makes antibodies that attack the thyroid follicles, which resynthesize and store thyroid hormones in a large protein called thyroglobulin these antibodies included, ant thyroglobulin (anti-TG) and anti-thyroid peroxidase (anti-TPO), which act as biochemical markers of HT illness(1)(2). Autoantibodies against TPO are found in more than 90% of HT patients, while (anti-TG) antibodies are found in approximately 80%(3). In addition, anti-TPO antibodies are found in about 10% of females, while other studies indicate that disease incidence is eight times higher in females than in males especially in elderly women, while Anti-TG antibodies are found in about 18% of elderly women (4). patients with various autoimmune disorders, such as primary adrenal hypofunction, Gravis disease, rheumatoid arthritis, pernicious anemia, gluten enteropathy, lupus disease, and type 1 diabetes, are more likely to develop Hashimoto's thyroid(5). Furthermore, many pathogeneses are linked to the interaction of hereditary and environmental variables(6). Additionally, the most prevalent cause of hypothyroidism is iodine deficiency; however, in areas where iodine usage is adequate, HT is the most common cause of hypothyroidism(7). T lymphocytes that are specific for thyroglobulin are created and sent to the thyroid

glands, where they release cytokines such *CTLA-4*which aid in the death of thyrocytes(8). When the immune system is passively attacking and breaking down the thyroid follicles in the early stages of Hashimoto's thyroiditis, elevated levels of triiodothyronine (T3) and thyroxine (T4) are produced in the peripheral blood by destroyed thyroid glands cells, simulating a transient hyperthyroid state(9). Thyroid autoimmunity has been related to a polymorphism in the TG gene, which codes for TG forms with various immunological activities(10). Genetic susceptibility to autoimmune thyroid disease had been well investigated and verified in twin and family investigations. The formation, progression, and severity of autoimmune thyroid disease have all been related to some genes (11). HLA, CTLA-4, PTPN type-22, VDR gene, thyroglobulin gene, and cytokines such as interferon - induced helicase1 gene IFIH1, are all believed to have a role in the HT disease(12). Antigen4 is a fundamental immunosuppressive cytokine that is mostly expressed on activated T cells(13), it is a protein receptor encoded by the CTLA-4 gene, which is situated on the long arm of chromosome 2 at position 33.2 (2q33.2). It has four exons and three introns, it belongs to the immunoglobulin superfamily and encodes a protein that sends an inhibitory signal to T lymphocytes(14). The protein contains a V domain, a Tran's membrane domain, and a cytoplasmic tail. Alternative variations of transcriptional splices have been identified, each of which codes for a different isoform. The membrane-bound isoform is a disulfide-bond entangled homodimer, while the soluble isoform is a monomer. It is converted into a peptide of 233 amino acids. The *CTLA-4* protein consists of a signal peptide and the main chain (the first 35 amino acids)(15). The most recent evidence of genome-wide interaction is that the *CTLA-4* gene is a key factor combining the environmental with genetic factors in the pathogenesis of multiple autoimmune illnesses including systemic lupus erythematosus (SLE), Hashimoto'disese, type I diabetes, psoriasis, and vitiligo(16).

## Patients and Methods

### Participants

A case-control study was applied to a total number of 200 females who were classified into 100 patients with Hashimoto's thyroiditis aged 18-68 years and 100 age and gender-matched healthy volunteers. Patients were recruited from Al-Sadder medical city in Al-Najaf Governorate, Iraq. All participants underwent medical examinations to make sure they were suffering from Hashimotos thyroiditis. The subjects included in this study were selected according to inclusion and exclusion criteria, inclusion criteria involved, firstly all patients who had been previously or recently diagnosed by a physician as having autoimmune hypothyroidism (Hashimoto's thyroiditis, in addition to these patients, have all clinical signs and symptoms of Hashimoto's thyroiditis, secondly all patients have a high level of thyroid antigen tests (thyroid peroxidase and thyroglobulin).

Exclusion criteria included thyroidectomy or under radioactive iodine treatment, non-thyroidal systemic disorders including acute and chronic hepatic, renal, cardiovascular, and cerebrovascular diseases, as well as benign and malignant tumors. and patients with other autoimmune illnesses including rheumatoid arthritis, diabetes, systemic lupus erythematous (SLE), and celiac disease.

***Measurements***

A venous whole blood sample of five milliliters was taken from each of the participants in this study. The blood was separated into two parts and labeled as follows: 3 mL of blood was placed in a gel activator tube, then subjected to centrifuge for ten minutes at 3000 Xg and divide into small aliquots (0.5 mL) for measuring thyroid function tests, anti-TPO and anti-TG antibodies. The remaining two milliliters of whole blood were collected in K3-EDTA tubes, then DNA was extracted and the extract was kept frozen at −80 °C to be used for genotyping of CTLA4 polymorphism (rs231775A/G) by real-time polymerase chain reaction (PCR). Polymorphism within *gene* CTLA4 (rs231775A/G) was done by real-time PCR and allelic discrimination assay utilizing a TaqMan probe, Applied Alpha DNA (Canada). The primers, probes, and Master Mix (80×) were also supplied by Promega (USA). The

TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. TaqMan® probes (quencher dye) and TaqMan® MGB probes. 0.2 µl Eppendorf tube in a total volume of 25mL using 2µg of genomic g DNA and a qPCR Master Mix GoTaq® Probe. The tubes were then placed in a thermal cycler, and heated at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

**Statistical analysis**

Results were tabulated and statistically analyzed by using a personal computer using MICROSOFT EXCEL 2010 and SPSS v. 26 (SPSS Inc., Chicago, IL, USA). Statistical analysis was done using: Descriptive: e.g. mean and standard deviation. Analytical: that includes: Chi-Squared ($\chi 2$), *t*-test. A value of P less than 0.05 was considered statistically significant.

**Results**

This case-control study was conducted on a total number of 200 females. These included 100 patients with 100 age and gender-matched healthy subjects as a control group. Patients were (100 %) female their age ranged from 18 to 68 years with a mean of 38.40 ± 11.18) years. Control subjects were also only women their ages ranged from 18 to 68 years with a mean of 39.25 ± 12.20 years, as shown (In table 1).

The p-values of body mass index (BMI) were calculated in patients with Hashimoto's thyroiditis and control groups, it was non-significant variation between both groups in the present study ($P<0.45$) and the mean ± SD was (30.21±5.32, and 29.75±5.70) respectively

Hashimoto's thyroiditis patients presenting with an aggressive form of hypothyroidism disease had significantly lower levels of T3, and T4, in addition to FT3 and FT4 ($p<0.000$) each other when compared with control who had normal values of these hormones.

The outcomes showed a significant increase in TSH, Anti TPO, and AntiTG levels in Hashimoto's thyroiditis patients compared with healthy control as shown in Table (1)

**Table (1): Levels (Mean ± SD) of some characteristics and functional thyroid tests in Sera of Studied Groups**

| Item | Group/No. | Mean ± SD | P value |
|---|---|---|---|
| **Age** | Control (100) | 38.4 ± 11.18 | 0.313 |
| | Patient (100) | 39.25 ± 12.20 | |
| **BMI** | Control (100) | 30.21 ± 5.32 | 0.45 |
| | Patient (100) | 29.75 ± 5.70 | |
| **T3** | Control (100) | 2.19 ± 0.41 | **<0.0001** |
| | Patient (100) | 1.47 ± 0.55 | |
| **T4** | Control (100) | 103.16 ± 20.75 | **<0.0001** |
| | Patient (100) | 62.96 ± 27.70 | |
| **TSH** | Control (100) | 2.66 ± 1 .15 | **<0.0001** |
| | Patient (100) | 35.96 ± 36.65 | |
| **fT3** | Control (100) | 4.98 ±0 .62 | **<0.0001** |
| | Patient (100) | 3.48 ± 1.18 | |
| **fT4** | Control (100) | 16.01 ± 7.58 | **<0.0001** |
| | Patient (100) | 9.94 ± 3.98 | |
| **AntiTPO** | Control (100) | 13.13 ± 8.03 | **<0.0001** |
| | Patient (100) | 337.81 ± 172.94 | |
| **AntiTG** | Control (100) | 29.96 ± 17 .53 | **<0.0001** |
| | Patient (100) | 1364.47 ± 1463.53 | |

In (figure 2) the frequency of the alleles of *CTLA4* SNP rs231775 (A/G) has mildly different, as it shows that the G allele is (0.551 %) in groups of patients while it is ((0.522%) in the control group, which means the G allele is more frequent in patients group than a control group.

On the other hand, the A allele is (0.472%) in the patient group and (0.485%) in the control group. That means the A allele is more frequent in the control group than in the patients group.

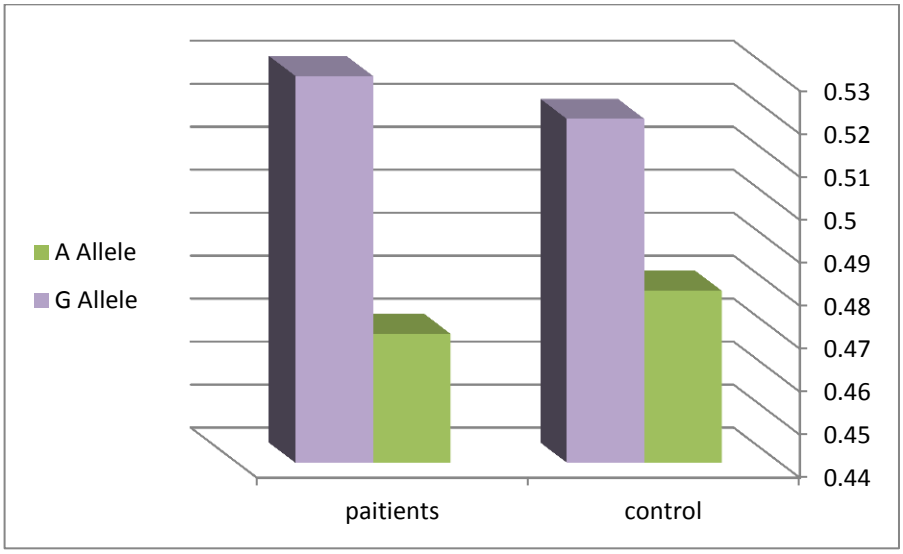


**Figure 2: The Percent Frequency of A&G alleles in Two Groups According to the HWE of *CTLA-4* SNP rs231775**

**Table2*: CTLA4* SNP rs231775 (A/G) analysis genotype for control according to the HWE.**

| Genotype of Control | Observed | Expected | Difference | $X^2$ | P value |
|---|---|---|---|---|---|
| **AA Reference** | 3 | 23.04 | 20.04 | 64.5 | 0.01 |
| **AG Heterozygote** | 90 | 49.92 | 40.08 | | |
| **GG Recessive** | 7 | 27.04 | 20.04 | | |
| **A allele%** | | | **48** | | |
| **G allele%** | | | **52** | | |

The Genotype frequencies of *CTLA4* SNP rs231775 (A/G) was not consistent with Hardy–Weinberg equilibrium in control persons as seen in (table2).

**Table3: *CTLA4* SNP rs231775 (A/G) analysis genotype for patients according to the HWE.**

| Genotype of patients | Observed | Expected | Difference | $X^2$ | P value |
|---|---|---|---|---|---|
| **AA Reference** | 2 | 22.09 | 20.09 | 65.045 | 0.01 |
| **AG Heterozygote** | 90 | 49.82 | 40.18 | | |
| **GG Recessive** | 8 | 28.09 | 20.09 | | |
| **A allele%** | | | **47** | | |
| **G allele%** | | | **53** | | |

The (table 3) shows the genotype frequencies of gene *CTLA4* SNP rs231775 (A/G) was not consistent with Hardy–Weinberg equilibrium in patients of HT.

**Table (4)**: **Genotype of *CYLA4* SNP rs231775 (A/G) in the studied groups.**

| SNP *CTLA4* | Control N=100 | Patients N=100 | Crude OR (CI 95%) P value | Adjusted OR (CI 95%) P value |
|---|---|---|---|---|
| **Codominant** | | | | |
| AA (Wild type) | 3 | 2 | 1 | |
| AG | 90 | 90 | 1.50 (0.25-9.20) 0.661 | 2.3 (0.34-16.4) 0.4 |
| GG | 7 | 8 | 1.7 (0.22-13.41) 0.61 | 2.8 (0.31-24.7) 0.36 |
| **Dominant** | | | | |
| AG+GG | 97 | 98 | 1.5 (0.25-9.30) 0.65 | 2.4 (0.34-16.60) 0.38 |
| **Recessive** | | | | |
| AA+AG (Wild type) | 92 | 93 | 1 | 1 |
| GG | 7 | 8 | 1.2 (0.40-3.13) 0.78 | 1.2 (0.4-3.5) 0.74 |
| **Additive** | | | | |
| 2AA+AG | 96 | 94 | 1 | |
| 2GG+AG | 104 | 106 | 1.041 (0.7030 -1.5413) 0.84 | |

In the current study, the allelic and genotypic distributions of *CTLA4* SNP rs231775 (A/G) were not significantly different between control subjects and HT patients for the AG genotype (OR=1.50, %95 CI: 0.25-9, 20, P=0.66); so there were (OR=2.3, %95 CI: 0.34-16.4, P=0.4) after adjustment for age, sex and BMI. While for GG (OR=1.7, %95 CI: 0.22-13.41, P=0.61) and there were find change after adjustment in age, sex and BMI (OR= 2.8, %95 CI: 0.31-24.7, P=0.36).

In addition to, same result obtained, there were no significant variation when compared healthy control and HT patients in dominant, recessive and additive models which examined by multinomial logistic regression analysis show **(Table 4)**

Finally, **(Table 5)** shows the biochemical characteristics of subjects with HT which studied according to the *CTLA4* SNP rs231775 (A/G) genotype. The biochemical features of (Age, BMI, T3, T4, TSH, fT3, fT4, TPO, Anti-TG) which relative to Codominant model of *CTLA4* SNP rs231775 (A/G) were studied by ANOVA test (table 5), while those of dominant model were examined by t- test **(Table 6).**The results in **(Table 5)** demonstrate a non-significant association of the Codominant model in T3, T4, TSH, fT3, fT4, TPO, and Anti- TG, the same result

obtained in dominant model in **(Table 6)** shows non-significant in all biochemical features which were studied, Age, BMI, T3, T4, TSH, fT3, fT4, TPO and Anti-TG.

**Table (5): Clinical distinctive of patient groups in proportion of *CTLA4* SNP rs231775 (A/G) genotype (Codominant model)**

| Clinical parameter | AA N=2 | AG N=90 | GG N=8 | P value |
|---|---|---|---|---|
| **Age** | 47.5 ± 13.4 | 38.4 ± 11.98 | 44.0 ± 11.5 | <0.271 |
| **BMI** | 27.8 ± 9.2 | 29.5 ± 5.7 | 33.03 ± 3.4 | <0.221 |
| **T3** | 1.678 ±0.73 | 1.47 ± 0.56 | 1.43 ± 0.47 | <0.864 |
| **T4** | 87.92 ± 40.85 | 62.02 ± 27.3 | 67.44 ± 30.9 | <0.384 |
| **TSH** | 30.34 ± 9.7 | 37.31 ± 37.2 | 22.2 ± 33.4 | <0.527 |
| **fT3** | 4.08 ± 0.16 | 3.46 ± 1.2 | 3.64 ± 1.15 | <0.716 |
| **fT4** | 9.88 ± 1.62 | 9.84 ± 4.1 | 11.14 ± 3.6 | <0.682 |
| **TPO** | 498.1 ± 189.4 | 338.65 ±171.4 | 288.26 ± 185.1 | <0.308 |
| **Anti-TG** | 2069.00 ±185.3 | 1349.1 ± 146.9 | 1361.58 ± 164.8 | <0.793 |

**Table (6): Clinical distinctive of patient groups in proportion** of ***CYLA4*** **SNP rs231775 (A/G) genotype (dominant model)**

| Clinical parameter | AA N=2 | AG+GG N=98 | P value |
|---|---|---|---|
| **Age** | 47.5 ± 13.4 | 38.84 ± 11.9 | <0.32 |
| **BMI** | 27.8 ± 9.24 | 29.7 ± 5.68 | <0.63 |
| **T3** | 1.6 ± 0.73 | 1.4 ± 0.56 | <0.61 |
| **T4** | 87.9 ± 40.85 | 62.4 ± 27.45 | <0.2 |
| **TSH** | 30.33 ± 9.68 | 36.0 ± 10.2 | <0.08 |
| **fT3** | 4.0 ± 0.16 | 3.4 ± 1.2 | <0.48 |
| **fT4** | 9.8 ± 1.62 | 9.9 ± 4.02 | <0.98 |
| **TPO** | 498.1 ± 189.4 | 334.5 ± 172.1 | <0.18 |
| **Anti-TG** | 2069.0 ± 185.3 | 1350.0 ± 120.2 | <0.49 |

## Discussion

The CTLA-4 (cytotoxic T lymphocyte antigen-4) gene has emerged as a key susceptibility locus for autoimmune endocrinopathies. The CTLA-4 gene, found on chromosome 2q33, encodes a co-stimulatory molecule found on the surface of activated T lymphocytes(17). The CTLA-4 molecule, along with CD28 (another costimulatory protein found on the surface of both resting and active T cells), is essential in T cell activation. T-cell reaction to antigen presentation T-cell activation occurs when antigen-specific cell-surface receptors are recognized. The T-cell receptor (TCR; CD3 complex) binds to the antigen, which is attached to an MHC class II molecule on the cell surface an antigen-presenting cell surface(18). +49A/G (rs231775), located in exon 1, is one of the most widely known polymorphisms in the CTLA-4 gene and causes a Thr>Ala amino acid substitution. This aberration hampers processes involving CTLA-4 molecules in the endoplasmic reticulum. Through this SNP, glycosylation of the CTLA-4 protein is reduced, leading to a decrease in cell surface expression of CTLA-4 protein.

Previous research discovered that the links between the development of autoimmune illnesses and viral infection may have a biological foundation, which is corroborated by genetic variants of *CTLA4*(17). The genotyping of *CTLA4* could cause the abnormal activation of the antiviral defense signaling pathway leading to the evolution of the autoimmune disease, such as in HT body substance and cell-mediated thyroid injury end up in the destruction of thyroid cells and hypothyroidism as a consequence. Previous GWAS on *CTLA4* identified

This polymorphism's relationship to other autoimmune illnesses has also been studied. Certain investigations have found a link between the GG genotype of A/G. rheumatoid arthritis in China(19). and celiac disease in Italy(20). However, in other

studies, no correlation was identified between the +49A/G SNP of CTLA-4 and autoimmune diseases, including HT in Italy(21). and Lebanon(21).

In the current study, the outcomes show no significant differences in allele or genotype frequencies for the rs231775 SNP between Hashimotos thyroiditis patients and control subjects. In addition, there was no significant difference between cases and controls concerning *CTLA4* genotype distribution with a predominance of AG and GG genotypes in studied cases. Indeed, there was a mild difference between cases and controls regarding *CTLA4* alleles 53% had a G allele while 47% had an A allele. While, in controls, 52% had a G allele while 48% of cases had an A allele. In studies conducted, results were obtained similar to those obtained in this study conducted by Mehrnaz Narooie - Nejad (22).

In contrast, another study carried out on rheumatoid arthritis disease included 1,200 samples in Chinese which identified significant associations observed between alleles of *CTLA4* (rs231775A > G, P = 0.007, OR = 1.17, 95%CI = 1.04–1.30), and RA disease susceptibility(19). Demonstrating the importance of CTLA-4 in the modulation of T cell responses, as well as the breakdowns in the B7-CD28/CTLA-4 pathway may alter T cell response and affect autoimmune diseases(23). In theory, reduced expression or function of CTLA-4 could lead to autoimmune T cell proliferation and contribute to the pathogenesis of autoimmune diseases such as HT(24).

The mechanisms by which *CTLA4* polymorphism contribute to HT pathogenesis remain to be explored, it is clear that the *CTLA4* gene locus has a role in the susceptibility of all autoimmune thyroid diseases such as HT. Single point mutation rs231775 at the CTLA4 gene may be a potential risk factor for HT susceptibility but in more sample size. In addition, *CTLA4* is a good candidate gene for autoimmune thyroid disease. Several studies carried out in different populations suggest that more than one *CTLA4* polymorphisms or SNP is related to this disease, that is mean not only dose rs231775 polymorphism affect the HT susceptibility but there are many other polymorphisms more effective on the HT susceptibility(10)(25).

In conclusion, current findings identified that fact; there is no association between rs231775 SNP of the *CTLA4*gene and the development of HT disease in the present study population, with the limitation of the present small sample size. Finally, more new and powerful research is needed to explain the role of *CTLA4*gene in AITD including HT disease.

## Abbreviations

TSH : Thyroid stimulating hormone ,TSHR:TSH receptor, HT : Hashimoto thyroiditis, AITD: Auto immune disease, , T3: Triiodothyronine, T4:Thyroxine , Anti TPO: Thyroid peroxidase anti gene ,Anti TG: Thyroglobulin anti gene ,fT3: Free T3, fT4:Free T4 , HT: Hashimoto's thyroiditis, (CTLA-4) genes: Cytotoxic T lymphocyte antigen-4, TG gene: Thyroglobulin gene, SLE Systemic lupus erythematous, BMI: Body mass index , GWAS: Genome-wide association studies ,AITD: Auto immune thyroid disease, T1DM :Type 1 diabetes mellitus, SLE :Systemic lupus erythematous, GD: Graves' disease , MS: Multiple sclerosis, RA :Rheumatoid arthritis, AAD: Autoimmune addison's disease,

**Consent to participate** All participants signed a written informed consent document.

**Author disclosures:** The authors declare that they have no conflict of interest.

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