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Exploring *DNMT1* Polymorphism and Expression in the Hashimoto Thyroiditis Pathogenesis

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Abstract

Background: Hashimoto thyroiditis is a chronic autoimmune disorder influenced by genetic and environmental factors. DNA methylation, regulated by DNA methyltransferase 1 (*DNMT1*), may play a critical role in its pathogenesis. This study investigated the association between *DNMT1* polymorphism, particularly rs2228611, and gene expression in Hashimoto thyroiditis patients and also compared serum levels of thyroid-stimulating hormone (TSH) and anti-thyroid peroxidase (anti-TPO) antibodies in both affected individuals and controls.

Methods: A case-control study of 100 participants (50 Hashimoto's thyroiditis patients and 50 controls) was conducted. TSH and anti-TPO levels were measured using the enzyme-linked immunosorbent assay (ELISA). *DNMT1* expression was analyzed via quantitative real time-polymerase chain reaction (qRT-PCR), while *DNMT1* (rs2228611 C/T) polymorphism was assessed by high-resolution melting-polymerase chain reaction (HRM-PCR).

Results: The results revealed that Hashimoto thyroiditis patients exhibited significantly elevated serum TSH and anti-TPO levels compared to healthy controls ($p < 0.0001$). *DNMT1* gene expression was upregulated by 1.7-fold in patients relative to controls ($p = 0.04$), suggesting a potential role in disease pathogenesis. Genotyping of *DNMT1* rs2228611 polymorphism revealed no significant differences in allelic or genotypic frequencies between groups. However, the TT genotype showed a non-significant trend toward increased disease risk ($p = 0.07$). The CT genotype appeared to confer a protective effect.

Conclusions: The study's findings suggest that elevated *DNMT1* expression and thyroid dysfunction are characteristic of Hashimoto thyroiditis, while the *DNMT1* rs2228611 polymorphism may have a limited but possible influence, warranting further study with larger cohorts.

Keywords: Anti-TPO, *DNMT1*, Gene expression, Hashimoto thyroiditis, SNP, TSH.

Introduction

Hashimoto thyroiditis is a chronic inflammatory disorder of the thyroid gland that affects approximately 0.8% of the population and may progress to chronic autoimmune hypothyroidism. This disease is characterized by decreased levels of thyroid hormones (T3 and T4), elevated thyroid-stimulating hormone (TSH) levels, and the presence of anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibodies, which are detected in approximately 95% and 60–80% of patients with Hashimoto thyroiditis, respectively (1,2), and the levels of these antibodies were increased abnormally (3). Thyroid follicles are being destroyed and replaced by tiny lymphocytes due to inflammation that significantly decreases the echogenicity of the thyroid parenchyma on the

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ultrasound image (4). The estimated prevalence of Hashimoto thyroiditis ranges from 0.3 to 1.5 per 1,000 people, with a pronounced gender disparity, as females are affected significantly more often than males, with a ratio between 7:1 and 10:1. Additionally, the disease demonstrates ethnic differences, occurring more frequently in individuals of White ancestry compared to those of Asian or Black descent. Pacific Islanders are infrequently affected, and the prevalence increases with age (5).

Genetic factors significantly affect the development of Hashimoto thyroiditis and involve both types of immune responses. The immune system's intolerance to thyroid autoantigens results in sustained infiltration by lymphocytes and the gradual degeneration of the thyroid tissue. Cytokines, especially those produced by TH1 cells, play a pivotal role in this inflammatory process by activating macrophages and contributing to follicular damage (6). The correlation between the inheritance of Hashimoto thyroiditis and HLA genes has been examined through the application of HLA serotyping and oligonucleotide DNA sequencing. HLA genes from different classes show varying degrees of association with Hashimoto thyroiditis across diverse ethnic groups (7). Hashimoto disease in Asians correlates with both HLA class 1 and class 2 genes, whereas in Caucasians, it is solely connected with the HLA class 1 gene (8).

In subjects that are hit with Hashimoto thyroiditis, autoantibodies against thyroid peroxidase and thyroglobulin are commonly found. These autoantibodies contribute to the autoimmune response (9). On the other hand, environmental factors such as excessive iodine intake, selenium deficiency, and gut microbiota alterations have been involved in the Hashimoto thyroiditis pathogenesis (10, 11). Microorganisms may also play a role by sharing epitopes with thyroid antigens, potentially inducing a chronic immune response against the thyroid gland (12).

The DNA methyltransferase 1 (*DNMT1*) gene is often situated on chromosome 19 at cytogenetic position 19p13.2 and contains 41 exons that encode a 1,616-amino acid protein. The *DNMT1* gene encodes an enzyme responsible for preserving DNA methylation profiles maintained throughout the process of DNA replication. DNA methylation, which involves the addition of a methyl group ($-CH_3$) to a cytosine residue in CpG dinucleotides, is a crucial epigenetic modification that regulates gene silencing, genomic imprinting, and chromosome stability (13,14). The rs2228611 is a synonymous single nucleotide variation in the *DNMT1* gene, depicted on chromosome 19 at position 10,156,401, within exon 17. This variation exists in three genotypes (CC, CT, and TT), corresponding to two alleles (C and T).

The present study investigated the association between *DNMT1* gene polymorphism and its expression levels in individuals with Hashimoto thyroiditis and determine whether the rs179247 variant is implicated for Hashimoto thyroiditis in individuals diagnosed with such a condition.

Materials and Methods

Study Population and Collection of Blood Sample

In line with the design of the assessment, it considered 100 participants, with about 50 individuals diagnosed with Hashimoto thyroiditis and 50 unaffected individuals as controls. The participants' ages ranged from 15 to 73 years. Blood specimens were collected from participants enrolled in the Endocrinology and Diabetes Specialty Center, Baghdad, Iraq, and private laboratories. The collection was done based on specialized criteria, while the analysis was conducted at the Iraqi Hereditary Company in Baghdad, Iraq, from April 2023 to April 2024.

Assessment of TSH and Anti-TPO Levels Using Enzyme-linked Immunoassay Assay

The enzyme-linked immunosorbent assay (ELISA) was assessed utilizing ELISA kits from DRG Inc., Germany for TSH, and from Demeditec Inc., Germany, for anti-TPO. The assay was based on the sandwich technique, where a monoclonal antibody, which selectively binds to a unique antigenic site on

the target molecule, was used to coat the microtiter wells.

RNA Extraction and Expression Analysis

Total RNA was obtained through extraction with TransZol Up Plus RNA reagent (TransGen Biotech, China). RNA concentration and purity were assessed using the NanoDrop OneC (Thermo Fisher Scientific, USA), and the total RNA samples were evaluated for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was converted to cDNA using the EasyScript® SuperMix kit (TransGen Biotech, China), in accordance with the manufacturer's protocol. Gene expression analysis for *DNMT1* and *GAPDH* was carried out via qRT-PCR on the Rotor-Gene Q system (QIAGEN, Germany) using the TransStart® Top Green qPCR SuperMix, and Ct values were recorded. Each reaction was performed in duplicate and the annealing temperature for the primers targeting *GAPDH* and *DNMT1* was 58 °C. Table 1 displays the primer nucleotide sequences used for amplification of *DNMT1* and *GAPDH* (15,16).

Table 1. The primer nucleotide sequences used for the study.

Target nucleotide	Direction	Nucleotide sequence	Annealing temperature
DNMT1	Forward	5`-GAGCTACCACGCAGACATCA-3`	56
	Reverse	5`-CGAGGAAGTAGAAGCGGTTG-3`	
GAPDH	Forward	5`-CGGGTTCCTATAAATACGGACTG-3`	56
	Reverse	5`-CCAATACGGGCCAAATCCGTTC-3`	
rs2228611 polymorphism	Forward	5`-GTGTGCCCCAAACATAATCC-3`	56
	Reverse	5`-TCTGGTTCAGCAAAACCAATC-3`	

DNA Extraction and HRM Analysis

Genomic DNA was extracted from blood specimens and quantified using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, USA). *DNMT1* (rs2228611 C/T) polymorphism was analyzed by HRM-PCR on the Rotor-Gene Q system (QIAGEN, Germany), employing the 2xTransStart Tip Green qPCR SuperMix kit for SNP sequence determination. The nucleotide sequences of primers for the rs2228611 polymorphism are shown in Table 1 (17, 18).

Statistical Analysis

Data were analyzed using IBM Statistical Package for Social Sciences (SPSS v29). One-way analysis of variance (ANOVA), t-tests, and chi-square tests assessed group differences, with significance set at 0.05 and 0.01. Odds ratios with 95% confidence intervals were used to evaluate the association of the *DNMT1* single nucleotide polymorphism (SNP) (rs2228611). Gene expression was quantified using the 2-ΔCt method (19-21).

Results

Demographics of the Study Population

In this study, 100 subjects in total were employed, 76% female and 24% male. The mean age of individuals with Hashimoto thyroiditis resulted in 44.92 ± 2.15 years, compared to 29.54 ± 1.49 years in the control group. This led to no differences that were significant between the two groups. Additionally, frequency as observed for females was higher than males in both the Hashimoto thyroiditis and normal control groups.

Assessment of TSH and Anti-TPO

Considering the recent evaluation, it has been revealed that the levels of TSH and anti-TPO in

the serum of Hashimoto thyroiditis patients were high in comparison to the normal control group of individuals. Essentially, it was noticed also that the mean ± standard error of TSH and anti-TPO levels in patients were 7.3±0.7 and 780.3±65.8, respectively, even though the values of the control group were 2.11 ± 0.2 and 331.9 ± 39.1, respectively. These findings indicated a significant increase in TSH ($p \leq 0.0001$) and anti-TPO ($p \leq 0.0001$) levels in infected individuals in relation to normal control group (Table 2).

Table 2. Comparison of patient and control groups in relation to TSH and anti-TPO.

Compound	Patient group	Control group	t-test	P-value
TSH (mIU/L)	7.3±0.7	2.1±0.2	7.2	0.0001**
Anti-TPO (mIU/mL)	780.3±65.8	331.9±39.1	5.1	0.001**

Data were expressed as mean + standard error of mean (SE); TSH: thyroid stimulating hormone; Anti-TPO: anti-thyroid peroxidase.

DNMT1 Gene Expression

The mean Ct values of *DNMT1* in individuals diagnosed with Hashimoto thyroiditis and age-matched unaffected normal controls were 22.1 and 22.4, respectively (Table 3). The mean Ct of *GAPDH* in patients was 20.2, while in the control group it was 19.7. The ΔCt values recorded were 1.9 for the Hashimoto's group and 2.7 for the control group, which showed that *DNMT1* gene expression was raised by 1.7-fold in patients, suggesting an increase in gene expression relative to healthy controls. Statistical analysis revealed a statistically significant alteration in gene expression between Hashimoto's patients' samples and control samples, with a p-value of 0.04. *DNMT1* gene amplification plots and dissociation curves are shown in Figure 1.

Table 3. *DNMT1* gene expression levels in the different study groups.

Parameter	Control	Patients
Means Ct of *DNMT1*	22.4	22.1
Means Ct of *GAPDH*	19.7	20.2
Mean of ΔCt	2.7	1.9
$2^{-\Delta Ct}$	0.2	0.3
Experimental/Control groups	0.157/0.157	0.259/0.157
Fold of gene	1.00	1.7
P-value	0.04*	

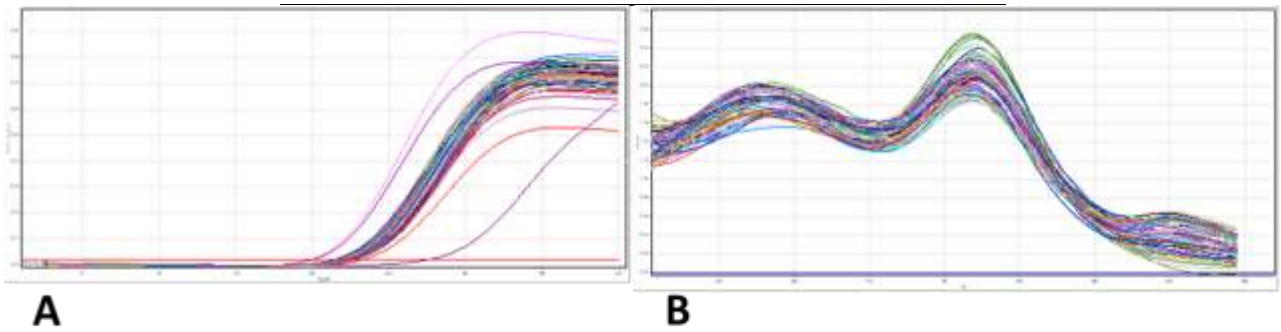

Fig. 1**.** The result of *DNMT-1* gene expression (A) *DNMT1* gene plots using qRT-PCR; (B) The *DNMT1* gene dissociation curves which were generated through qRT-PCR (melting temperatures ranged from 77 °C to 78 °C).

Genotyping by HRM

The genotypic and allelic frequencies were assessed in both the patient and control populations, with the wild type serving as the baseline. Table 4 shows a distribution that did not differ significantly in genotypic and allelic frequencies (rs2228611) between Hashimoto thyroiditis patients and control groups. A negative association was observed with the CT genotype in correlation to Hashimoto thyroiditis, with an odds ratio and a p-value of 0.9, indicating it could serve the purpose of protection. In contrast, the TT genotype exhibited a p-value of 0.07, which was not statistically significant. Similarly, the T allele pointed to no difference that can be adjudged statistical in comparing patients and controls, as indicated by a p-value of 0.2.

Table 4. Genotypic and allelic frequencies according to Hardy-Weinberg equilibrium.

Genotype (rs2228611)	CC	CT	TT	Total		C	T
Patient	(16) 32%	(28) 56%	(6) 12%	50	Allele Frequency	60%	40%
Control	(18) 36%	(32) 64%	(0) 0%	50		68%	32%
P-value	--	0.9	0.07	--		--	0.2
Odds Ratio	1.00	0.9	15.5	--		1.00	1.4
Confidence Interval [95%]	Reference	0.424 to 2.287	0.761 to 279.05			Reference	3.13 to 2.53

Discussion

Hashimoto thyroid disorder is a prevalent autoimmune condition that affects individuals with a genetic predisposition, influenced by various factors (22). The incidence of Hashimoto thyroiditis increases with age, with the highest occurrence observed in individuals aged 41 to 50 years, followed by those aged 31 to 40 years (23). Research has demonstrated that serum concentrations of T3 and T4 decrease among people identified with Hashimoto thyroiditis. This decrease can be accompanied by a significant increase in TSH levels. Elevated TSH levels are indicative of a thyroid disorder, with a high incidence of Hashimoto thyroiditis. Several studies have reported similar findings (5). The results of Omer *et al*. (24) indicate that the standard error of the mean for TSH levels in cases was significantly higher compared to control levels, supporting the findings of the current study. Additionally, research conducted by AL-Badri *et al*. (25) found that both T3 and T4 levels were lower in individuals with Hashimoto thyroiditis, while TSH levels were elevated. The studies suggest that elevated TSH levels accompanied by reduced concentrations of T3 and T4 hormone levels may indicate the presence of Hashimoto thyroiditis, a disease in which one's immunity mistakenly interferes and damages thyroid cells. The destruction of thyrocytes, which produce thyroid hormones (TH), is caused by cytotoxic T cells, leading to the death of thyroid cells and a subsequent decrease in the production of T3 and T4 (4). On the other hand, the loss of thyroid cells produced causes the destruction of TSHR (thyroid-stimulating hormone receptors), resulting in an increase in TSH in the blood and a drop in T3 and T4 (26).

According to a study presented by Sviridonova and colleagues (27), TSH levels varied between morning and afternoon. When it was morning, the TSH levels were 5.83 mU/L above the normal range (2.5-4.0 mU/L). However, during the afternoon period, the TSH level usually gets to 3.79 mU/L, which

falls within the normal threshold. These findings align with the expected outcomes of Hashimoto thyroiditis pathophysiology. This condition involves a situation whereby the lymphocyte infiltrates the thyroid, followed by the generation of antibodies targeting those antigens that are peculiar to the thyroid, such as thyroid peroxidase and thyroglobulin. As a result, the levels of these thyroid hormones in the blood decrease. This reduction stimulates the pituitary gland to secrete more TSH, causing TSH levels in the serum to rise (28). The findings are closely associated with those of Fadhil *et al*. (29), who noted that thyroid autoantibodies, including anti-TPO, anti-Tg, and anti-TSH receptor antibodies, are widely available and commonly used in clinical diagnostic laboratories. Anti-TPO antibodies are the predominant autoimmune antibodies directed at the thyroid in Hashimoto's disease. Elevated levels of anti-TPO antibodies have been linked to the clinical signs of disease development in the future (30). According to Ralli *et al*. (9), elevated levels of anti-TPO are associated with 95% of Hashimoto thyroiditis cases.

Previous studies on autoimmune Graves' disease patients conducted by Saban *et al*. (31) found that newly diagnosed patients exhibited elevated levels of *DNMT1* mRNA expression compared to individuals with other thyroid diseases. This increase in expression may be linked to abnormal alterations in the methylation of immune regulatory genes, which may influence the development of autoimmunity. On the other hand, the patients receiving methimazole treatment experienced a reduction in *DNMT1* expression levels that matched those of healthy individuals. This demonstrates that treatment can reverse the upregulation and lower the genomic expression of *DNMT1* (31). In a previous study, Guo *et al*. (32) found that patients newly diagnosed with Graves' disease exhibited downregulated *DNMT1* mRNA expression in B and T lymphocytes. Following treatment, there was an upward trend in *DNMT1* expression in these patients. This indicates that treatment may help normalize *DNMT1* expression levels, although the study observed variability in global methylation changes among patients. A study by Kyono *et al*. (33) found that thyroid hormones do not directly regulate *DNMT1* itself, but they do influence the levels of other DNA methyltransferases like DNMT3a. For example, in mouse neuronal cells, the thyroid hormone (T3) directly raises the expression level of DNMT3a.

The previous study found no association between the rs2228611 variant and Graves' disease in the Polish-Caucasian number of individuals. Additionally, no differences were observed in the allele frequencies of these variants among Graves' disease patients categorized by specific disease (34). It has been observed that the genotypic and allelic frequencies of the *DNMT1*+32204A/G polymorphism did not significantly differ between healthy controls and patients with AITD. However, a greater prevalence of the G allele and GG genotype of this SNP was noted in individuals that have intractable Graves' disease compared to those that have Graves' ailment in remission. The *DNMT1*+32204GG genotype is associated with DNA hypomethylation and has been linked to the intractability of Graves' disease (35). A study by Cai *et al*. (36) suggests that rs2228611 may play a role in the genetic susceptibility to AITD, potentially through mechanisms involving DNA methylation and immune regulation. Additionally, it has been suggested that rs2228611 in *DNMT1* may be linked to schizophrenia that tends to exhibit positive symptoms. The *DNMT1* gene may influence the clinical symptoms of schizophrenia by regulating the gene expression implicated in the dopaminergic and GABAergic systems (37). The results revealed that the *DNMT1* rs2228611 SNP is linked to an elevated risk of ovarian cancer in Polish women, and it has been revealed that patients were more likely to have the AG genotype at rs2228611 compared to the control. Thus, the rs2228611 AG genotype may represent a potential risk factor for breast cancer (38).

Based on the findings, it appears that patients have significantly elevated anti-TPO and TSH levels in comparison to the untreated category. In essence, this portends that there might be a thyroid dysfunction or other underlying medical condition in the patient group. The findings revealed that the *DNMT1* gene is highly expressed in patients compared with the standard control group. Hashimoto thyroiditis may be exacerbated by the *DNMT1* gene's overexpression, which alters methylation patterns of genes responsible for immune function and thyroid hormone regulation. This could potentially lead to abnormal immune responses and thyroid disorder. *DNMT1* gene polymorphisms rs2228611 showed that the T allele and TT genotype may be associated with a higher risk of Hashimoto thyroiditis. Although the higher prevalence of the TT genotype in patients compared to controls, its potential as a risk factor remains inconclusive due to the lack of statistical significance. This non-significance may be a result of the small sample size and random sample selection. According to this investigation, patients with the TT genotype were at a higher risk of developing Hashimoto thyroiditis than individuals with the CC genotype. This suggests a potential association with Hashimoto thyroiditis. While the odds ratio (OR) indicates a strong association between the TT genotype and thyroiditis caused by Hashimoto's, is essential to account for the study's limitations, including the limited sample size and need for replication in larger studies.

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Ethical Consent
The Ethics Committee of the Department of Biology, College of Science, University of Mastansiriyh, Baghdad, Iraq, approved this study, as did the Iraqi Ministry of Health, with the approval number BCSMU/1023/0051Z.

Conflict of interest
The authors declare that they have no conflicts of interest.

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Authors contribution
H.J.M. conceived and designed the study. Z.S.S. performed the molecular experiments. A.D.A.A. contributed to clinical sample collection. All authors contributed to data analysis, drafted the manuscript, and approved the final version.

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