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The CpG Island Methylation Status and mRNA Expression Level of *CTLA4* in Childhood Hashimoto's Thyroiditis

Fatemeh Safari[1,2,3], Mohammad Hossein Ahmadi[4], Mehdi Azad[4], Neda Karami[5], Amirhosein Maali[5,6], Neda Mohammadi[7], Ali Homaei[8], Farshad Foroughi[9,10]

[1]Department of Pediatrics, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[2]Children Growth Research Center, Qazvin University of Medical Sciences, Qazvin, Iran
[3]Research Institute for prevention of Non-communicable Diseases, Qazvin University of Medical Sciences, Qazvin, Iran
[4]Department of Medical Laboratory Sciences, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[5]Department of Medical Biotechnology, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[6]Department of Immunology, Pasteur Institute of Iran, Tehran, Iran
[7]Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[8]Department of Medicine, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[9]Department of Immunology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[10]Cellular and Molecular Research Center, Research Institute for Prevention of non-Communicable Disease, Qazvin University of Medical Sciences, Qazvin, Iran

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Corresponding Author: Farshad Foroughi, Cellular and Molecular Research Center, Research Institute for Prevention of non-Communicable Disease, Qazvin University of Medical Sciences, Qazvin, Iran
Phone: +98 912 582 24 76 **E-mail:** farshad295@yahoo.com **ORCID:** orcid.org/0000-0002-6939-6942

Abstract

Objective: Hashimoto's thyroiditis (HT) is an autoimmune thyroid disease characterized by thyroid-specific autoantibodies. Recent studies have shown the critical footprint of DNA methylation in autoimmune diseases. The aberrant DNA methylation of *CTLA4* has been previously reported in autoimmune thyroid diseases. This study aimed to investigate the methylation status of the CpG island of *CTLA4* promoter and its mRNA expression level in HT patients.

Materials and Methods: In this case-control study, 45 HT patients (admitted to Qods Hospital, Qazvin, Iran) and 5 healthy individuals participated. After RNA and DNA extractions, the DNA methylation pattern of the CpG island of *CTLA4* promoter and *CTLA4* mRNA expression level were evaluated. All statistical analyses were performed using SPSS ver 20.

Results: Our results indicated partial hypermethylation status in the CpG island of *CTLA4* promoter in HT patients compared to normal individuals, but this hypermethylation was not significantly higher (p=0.332). The mRNA expression level of *CTLA4* was significantly decreased in HT patients compared to that of controls (Foldchange=0.31, p=0.015). Also, serum level of anti-TPO antibody was not significantly correlated with *CTLA4* expression level and its methylation status.

Conclusion: Since the *CTLA4* acts as an immune checkpoint that leads to the downregulation of immune response, partial hypermethylation and downregulation of *CTLA4* may play a critical role in preventing switching off the immune response after a hyperactivation against the thyroid.

Keywords: Autoimmune diseases, Hashimoto disease, *CTLA4*, DNA methylation, gene expression

ORCID: F. Safari 0000-0003-3943-8803, M.H. Ahmadi 0000-0002-3414-5019, M. Azad 0000-0002-5818-213X, N. Karami 0000-0001-5681-2794, A. Maali 0000-0002-0699-3010, N. Mohammadi 0000-0002-2542-4518, A. Homaei 0000-0001-5926-5482, F. Foroughi 0000-0002-6939-6942

Introduction

Hashimoto's thyroiditis (HT) is an autoimmune thyroid disease characterized by thyroid-specific autoantibodies leading to increase in the thyroid gland volume, lymphocyte infiltration of parenchyma, and hypothyroidism (1). In 1912, Haraku Hashimoto introduced this disease (2). The prevalence of HT is about 30 to 150 per 100.000 individuals worldwide (3). About 10% of women have a significant level of thyroid-specific autoantibodies, and 2% of women manifest the clinical symptoms of HT (3). Also, studies have shown a significant correlation between age and race; higher age and white race are related to higher prevalence of HT (3).

Environmental factors, genetic susceptibility, and epigenetics factors are three primary etiologies of HT (4). Recent studies have shown the critical footprint of DNA methylation in autoimmune diseases (5-7). DNA methylation in the CpG island of promoters inhibits the mRNA expression level. Aberrant DNA methylations are seen in various diseases, e.g., HT. The aberrant DNA methylation pattern is seen in *PTPN22* (8), *IL2RA* (9), *ICAM-1* (10), in HT patients.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is an immune receptor responsible for the downregulation of immune responses expressed on the surface of activated T-cells (11). CTLA4 is a homolog of CD28, a T-cell co-stimulatory protein. CD28 stimulates the immune response by binding to CD80/CD86. In T-cell activation, the surface expression of CTLA4 increases and binds to CD80/CD86 on the surface of antigen-presenting cells (with more affinity compared to CD28), which leads to switching-off of the immune response by transmitting inhibitory signals into T-cells (11-13). Haploinsufficiency of *CTLA4* is observed in *CTLA4* haploinsufficiency with autoimmune infiltration (CHAI) disease, which may lead to lymphoproliferation, autoimmunity, recurrent infections, and lymphoma (14). The aberrant DNA methylation of *CTLA4* is previously reported in autoimmune thyroid diseases. In a genome-wide analysis of DNA methylation, Limbach et al. (15) reported that the CpG island of CTLA4 promoter was hypermethylated in Graves' disease. Moreover, *CTLA4*-associated DNA polymorphisms are reported in autoimmune thyroid diseases (16).

In this study, we aimed to investigate the methylation status of CpG island of *CTLA4* promoter, *CTLA4* mRNA expression level, and their correlation in HT patients. In addition, we evaluated the possible correlations with anti-thyroperoxidase antibodies (anti-TPO).

Materials and Methods

Participants and Sampling

In this case-control study, 45 HT patients (admitted to Qods Hospital, Qazvin, Iran) and 5 healthy individuals were included. The exclusion criteria were as follows: Being over the age of 15 years, receiving any thyroid medications, having secondary metabolic disease, negative anti-TPO, and personal desire to leave the study with informed consent. This study was approved by the ethics committee of Qazvin University of Medical Sciences, Qazvin, Iran (IR.QUMS.REC.1398.077).

For sampling, 7.5 mL of whole blood was collected from each participant; 5 mL was collected into the anti-coagulant tube (immediately transferred to the laboratory for molecular assays), and 2.5 mL was collected into the clot tube (for anti-TPO evaluation). Serum was separated, and anti-TPO was evaluated in the serum of participants by Cobas e411 analyzer (Roche, Germany). All samplings were performed before medication (levothyroxine).

DNA Extraction and Bisulfite Treatment

The DNA extraction was performed using GeneAll kit (Southern Korea) as manufacturer protocol. The extracted DNA samples were treated by EpiTect Fast DNA Bisulfite Kit (Qiagen, USA) to substitute unmethylated cytosine residues with uracil.

Methylation-specific PCR for CTLA4 CpG-island

[Methylation-specific polymerase chain reaction (PCR) - MSP] was used through methylated and unmethylated primers to amplify CpG island of the *CTLA4* promoter. For this aim, 10 µL of 2x master mix (Ampliqon, Denmark), 1 µL of each primer (Table 1), and 1 µL of bisulfite-treated DNA were mixed and raised to 20 µL using ddH_2O. Thermal cycles were performed as follows: 1) pre-denaturation stage for 10 min in 95°C, 2) 38 thermal cycles including denaturation for 15 sec in 94°C, annealing for 30 sec in 53°C, and extension for 15 sec in 72°C, and 3) final extension for 10 min in 72°C, using applied biosystems (ABI) thermal cycler (Thermofisher, USA). To detect the methylation patterns, the amplicons were loaded on 1% agarose gel. Positive and negative control of methylation was used to verify the accuracy of the MSP.

RNA Extraction and cDNA Synthesis

The GeneAll RNA extraction kit (Southern Korea) was used for the extraction of total RNA. Then, extracted RNAs were reversely transcribed to cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis kit (USA). cDNAs were stored at -80°C for the next steps.

Real-time PCR

Real-time PCR was used for the evaluation of *CTLA4* mRNA expression levels in HT patients and healthy participants. For this aim, 10 µL of SYBR™ Green PCR Master Mix (Thermofisher, USA), 0.4 µL of each primer

(Table 1), and 2 µL of cDNA were mixed and raised to 20 µL using ddH_2O. Real-time PCR stages were performed as follows: 1) pre-denaturation for 15 min in 95°C, 2) denaturation for 19 sec in 95°C, 3) annealing for 19 sec in 61.5°C, and 4) extension for 30 sec in 72°C, using the Rotor-Gene Real-time Thermal cycler (QiaGen, USA). *GAPDH* gene was used as the internal control. The fold change was calculated using the *Pfaffl* method.

Statistical Analysis

All statistical analyses were performed by SPSS software ver 20. Significant level was considered when p value was <0.05. Also, Ct values of real-time PCR results were calculated using REST software.

Results

Demographical Results

Among 45 patients, 36 individuals (80%) were female, while 2 individuals (60%) were female in controls (n=5). The frequency of sex was not statistically different between patients and controls. The mean age of HT patients was 12.63 ± 4.21 years, while the mean age of the controls was 11.75 ± 4.32 years (p=0.719). The BMI values of HT patients (median=21.150 kg/m^2) were significantly higher than those of control individuals (16.370 kg/m^2, p=0.041).

Methylation Status and Expression Level of *CTLA4*

Our results showed no significant difference in CpG island methylation status of *CTL4* between HT patients and controls. Among 45 patients, CpG island methylation of *CTL4* was hemimethylated in 17 (34%) patients and methylated in 33 (66%) patients. On the other hand, in healthy individuals, CpG island methylation of *CTL4* was hemimethylated in 3 (60%) individuals and methylated in 2 (40%) individuals. Our results indicated partial hypermethylation status in the CpG island of *CTLA4* promoter in HT patients compared to normal individuals, but this hypermethylation was not statistically significant (p=0.332) (Figure 1).

The mRNA expression level of *CTLA4* was significantly decreased in HT patients compared to controls (p=0.015). The median ΔCt of *CTLA4* in HT patients was 3.550, while it was 1.660 in controls (Table 2). Foldchange of *CTLA4* expression was estimated as 0.31 in Hashimoto's patients, compared to a healthy status.

Logistic regression analysis showed that the expression level of *CTLA4* was reversely correlated with the odds of HT. For every unit increase in ΔCt of *CTLA4*, the odds of disease increased by 60% (borderline significance, p=0.061). Also, CpG island methylation of *CTL4* was not statistically correlated with *CTLA4* expression (Table 3).

Correlation of Anti-TPO with Methylation Status and Expression Level of *CTLA4*

The serum anti-TPO level was significantly higher in HT patients (210.35 ± 317.97 IU/mL) compared to healthy individuals (5.50 ± 4.27 IU/mL, p<0.001). Moreover, the qualitative anti-TPO analysis showed that 60% of patients (27 of 45 individuals) were anti-TPO positive, while all healthy controls were negative for anti-TPO antibody (p=0.013). Linear regression for $\log_{10}$ anti-TPO antibody titer showed that anti-TPO antibody titer was not significantly correlated with *CTLA4* expression level, methylation status, BMI, sex, and age (Table 4).

Discussion

The results of our study showed that a partial hypermethylation was observed in the CpG island of *CTLA4* promoter in HT patients compared to normal individuals, but this hypermethylation was not statistically significant. The mRNA expression level of *CTLA4* was reduced in HT patients compared to controls. However, our results did not show a statistically significant correlation between *CTLA4* expression and methylation.

Recent studies have shown that aberrant DNA methylation has a critical impact on autoimmune disease (17). CTLA4 is an immune checkpoint molecule that leads to immune suppression when activated in T-cell response

Table 1. Oligonucleotide sequences used for MSP and real-time PCR of *CTLA4* gene

Assay	Target	Order	Sequence
MSP	Methylated primers	Forward	5-AATTTTAAGTGTATAGAATTTCGG-3
		Reverse	5-ATTCAAAAAATTAAAACCGTC-3
	UnMethylated primers	Forward	5-TAATTTTAAGTGTATAGAATTTTGG-3
		Reverse	5-ATTCAAAAAATTAAAACCATC-3
Real-time PCR	CTLA4	Forward	5-GTAATTGATCCAGAACCGTGCC-3
		Reverse	5-CACATTCTGGCTCTGTTGGG-3
	GAPDH	Forward	5-CAA TGA CCC CTT CAT TGA CC-3
		Reverse	5-TGG AAG ATG GTG ATG GGA TT-3

MSP: Methylation-specific PCR, PCR: Polymerase chain reaction

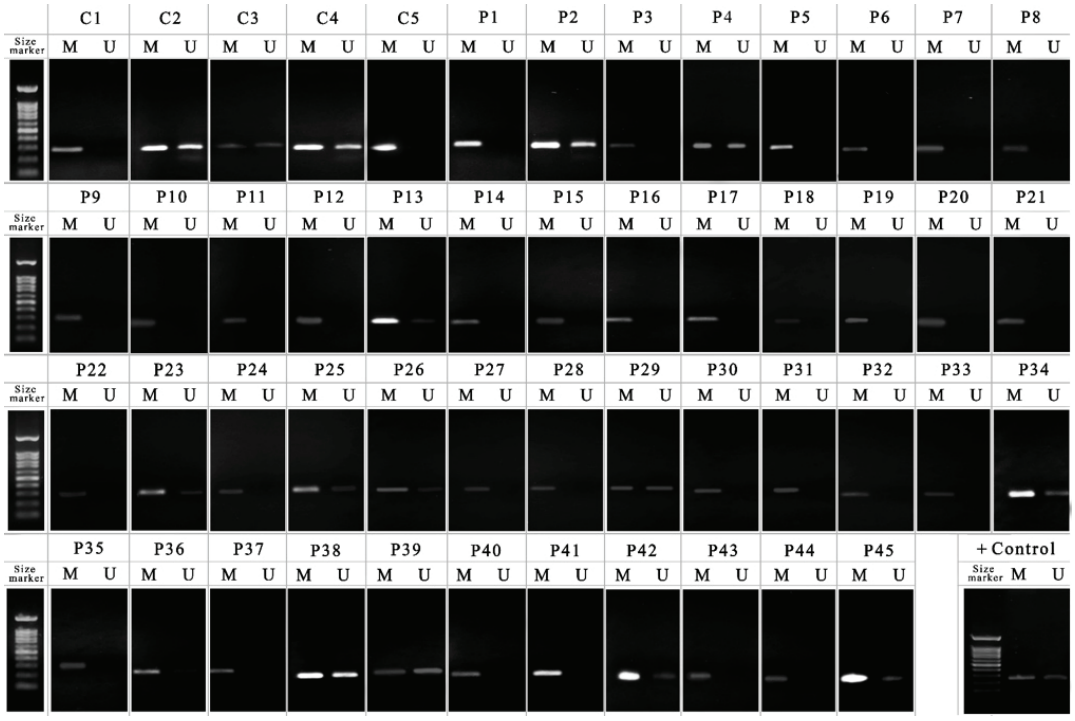

Figure 1. Methylation pattern of *CTL4* CpG island in Hashimoto's thyroiditis patients and controls. Among 45 patients, CpG island methylation of *CTL4* was hemimethylated in 17 (34%) and methylated in 33 (66%), while CpG island methylation of *CTL4* was hemimethylated in 3 (60%) patients and methylated in 2 (40%) individuals.
M: Methylated primer, U: Unmethylated primer, C: Control, and P: Patient

Table 2. Evaluation of differences in parameters for case/control groups

Variable		Control (n=5)	Case (n=45)	p-value*
ΔCt of qRT-PCR; Median (IQR)		1.660 (1.150, 1.700)	3.550 (2.140, 4.000)	**0.015**
Anti-TPO antibody (quantitative); Mean ± SD		5.50 ± 4.27	210.35 ± 317.97	**<0.001**
Age; Mean ± SD		11.75 ± 4.32	12.63 ± 4.21	0.719
BMI; Median (IQR)		16.370 (14.710, 18.047)	21.150 (18.080, 23.010)	**0.041**
Anti-TPO antibody (qualitative)	Negative	5 (100%)	18 (40%)	**0.013**
	Positive	0 (0%)	27 (60%)	
Sex	Male	2 (40%)	9 (20%)	0.564
	Female	3 (60%)	36 (80%)	
Methylation	Hemi-methylated n (%)	3 (60%)	14 (31.11%)	0.332
	Methylated n (%)	2 (40%)	31 (68.89%)	

IQR: Interquartile range, SD: Standard deviation; *The p-value of categorical variable was estimated using the Monte-Carlo simulation, PCR: Polymerase chain reaction, BMI: Body mass index, TPO: Thyroperoxidase antibodies

(18). Recent studies have shown the role of aberrant DNA methylation of *CTLA4* in Greaves' disease, an autoimmune thyroid disease (15,19). Also, HT is an autoimmune disease corresponding to attacking the immune system to the thyroid (8,20). The investigation of possible molecular etiology on HT provided significant data regarding the involvement of aberrant DNA methylation in HT (8,20). This study aimed to investigate the DNA methylation pattern in the CpG island of *CTLA4* in HT disease.

In this case-control study, we used HT patients who did not receive any medications, e.g., levothyroxine. Therefore, we desired accessibility on the intact epigenome profile of HT. Our results showed that 60% of healthy individuals displayed hemimethylated state in *CTLA4* CpG island, while in HT patients, 34% of participants had hemimethylated status in CpG island. Also, 66% of HT patients had methylated CpG island in *CTLA4* promoter, and 40% of healthy individuals had methylated CpG island statues in

Table 3. The effects of different factors on Hashimoto's thyroiditis using Logistic Regression

Variable	Multiple Analysis		Univariate Analysis	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age	0.91 (0.71, 1.19)	0.504	1.04 (0.84, 1.29)	0.724
BMI	1.36 (0.96, 1.9)	0.084	1.33 (0.99, 1.78)	0.058
Methylation (methylated vs non-methylated)	1.85 (0.15, 22.87)	0.614	2.61 (0.48, 14.3)	0.259
PCR positivity	1.6 (0.71, 3.67)	0.257	1.62 (0.98, 2.7)	0.061
Interaction of PCR: Methylation (methylated)	0.93 (0.43, 2.05)	0.844	-	-
Sex (female vs male)	1.1 (0.12, 9.71)	0.934	2.14 (0.38, 11.82)	0.384

OR: Odds ratio, CI: Confidence interval, Hemi-methylated and male were set as the reference levels of their variables, PCR: Polymerase chain reaction, BMI: Body mass index

Table 4. The effects of different variables on $\log_{10}^{\text{anti-TPO}}$ antibody titer using Linear regression

	Multiple Analysis		Univariate Analysis	
	Beta (95% CI)	p-value	Beta (95% CI)	p-value
Age	-0.01 (-0.08, 0.07)	0.885	0.02 (-0.04, 0.08)	0.469
BMI	0.06 (-0.03, 0.14)	0.185	0.04 (-0.02, 0.11)	0.155
Methylation (methylated vs non-methylated)	0.64 (-0.61, 1.86)	0.324	0.39 (-0.08, 0.86)	0.119
PCR positivity	0.02 (-0.27, 0.32)	0.873	0.03 (-0.11, 0.17)	0.675
Interaction of PCR: Methylation (methylated)	-0.09 (-0.44, 0.27)	0.634	-	-
Sex (female versus male)	0.26 (-0.37, 0.87)	0.429	0.34 (-0.22, 0.89)	0.24

CI: Confidence interval, PCR: Polymerase chain reaction, BMI: Body mass index, Hemi-methylated and male were set as the reference levels of their variables

CTLA4 promoter. This finding has established the CpG island promoter of *CTLA4* is partially hypermethylated, but it is not statistically significant. Limbach et al. (15) reported that *CTLA4* was hypermethylated in Graves' disease patients compared to healthy individuals. De Vos et al. (21) reported higher levels of CpG island methylation of *CTLA4* promoter in papillary thyroid carcinoma compared to normal adjacent tissues.

The mRNA expression level of *CTLA4* was significantly lower in HT patients compared to controls (p=0.015). As mentioned above, *CTLA4* CpG islands were partially hypermethylated in HT compared to healthy controls. These findings suggest that the expression level of *CTLA4* is associated with the methylation status of the CpG island of this gene. On the other hand, the haploinsufficiency of *CTLA4* was observed in autoimmune diseases, e.g., CHAI disease (14). Our findings show that the lower expression of *CTLA4* is associated with HT disease as another autoimmune disease. As mentioned above, CTLA4 acts as a downregulatory factor of immune response after activation. Since HT is an autoimmune disease, there is hyperactivation of the immune system against the thyroid. Therefore, the reduced mRNA expression level of *CTLA4* in HT may be associated with the deletion of immune checkpoints and avoiding immune suppression. Probably, this event suggests hyperactivation of the immune system against the thyroid. Regarding our study, Tokić et al. (22) and Kucharska et al. (23) showed that *CTLA4* mRNA expression level was lower in HT patients compared to healthy individuals. Wojciechowska-Durczynska et al. (24) found that mRNA of *CTLA4* was overexpressed in children with autoimmune thyroiditis compared to adults with autoimmune thyroiditis. In addition, de Vos et al. (21) observed that the mRNA expression level of *CTLA4* was reduced in papillary thyroid carcinoma compared to normal adjacent tissues.

Study Limitations

The relatively low sample size of healthy individuals is the limitation of our study. For further studies, we suggest comparing medication-naive HT patients and levothyroxine-received HT patients to investigate the role of DNA methylation of CpG island of *CTLA4* promoter on larger sample size. Also, a genome-wide analysis of the DNA methylome of HT patients helps to investigate the methylome-transcriptome network of HT to clarify HT's molecular etiology.

Conclusion

Finally, our results have shown that the mRNA expression of *CTLA4* is lower in HT patients than healthy controls and may be related to the partially hypermethylated status of CpG island of *CTLA4* promoter in HT patients. Since *CTLA4* acts as an immune checkpoint that leads to the downregulation of immune response, partial

hypermethylation and downregulation of *CTLA4* may play a critical role in preventing switching off of the immune response after a hyperactivation against the thyroid gland.

Ethics

Ethics Committee Approval: This study was approved by the ethics committee of Qazvin University of Medical Sciences, Qazvin, Iran (IR.QUMS.REC.1398.077).

Informed Consent: Informed consent forms were obtained from the patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.F., Concept: M.H.A., A.H., M.A., Design: F.F., A.H., Data Collection or Processing: F.S., Analysis or Interpretation: F.S., A.M., Literature Search: N.M., Writing: N.K.

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