Association of CTLA-4 gene polymorphisms -318C/T and +49A/G and Hashimoto's thyroidits in Zahedan, Iran

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Abstract. Hashimoto's thyroiditis (HT) is a chronic inflammation of the thyroid gland and is known as the most common autoimmune disease. Development of autoimmune destruction of thyroid cells is a multi-step process involving convergence of genetic and environmental factors. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) has an important role in homeostasis and negative regulation of immune responses, and is therefore considered to be a key element in the development of autoimmune diseases. The present study evaluated the association of the CTLA-4 gene polymorphisms 318C/T (rs5742909) and +49A/G (rs231775) with HT in an Iranian population (including 82 patients with HT and 104 healthy controls who were referred for routine premarital blood screenings). Genotyping was performed using the tetra-primer amplification refractory mutation system polymerase chain reaction technique. No significant differences were observed in genotype and allele frequencies in the single nucleotide polymorphisms (SNPs) between cases and controls. In the cases as well as in the controls, the TT genotype in the -318C/T polymorphism was absent and the predominant genotype was CC, while the predominant genotype for the +49A/G SNP was AA. As only few studies in this field have assessed Iranian and even Middle Eastern populations, additional studies with a higher number of samples are recommended to further assess the impact of -318C/T (rs5742909) and +49A/G (rs231775) polymorphisms of CTLA-4 on HT.

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Introduction

Hashimoto's thyroiditis (HT) is a chronic inflammation of the thyroid gland, which was first described by Hashimoto as Struma lymphomatosa in 1912 (1). At present, HT is the most common type of autoimmune disease, endocrine disorder and hypothyroidism (2-4). Approximately 2% of the general population are affected by HT with a high prevalence in middle-aged individuals and in women (5,6).

In HT, thyroid cells are lost, leading to severe damage of the thyroid gland and subsequent reduction in thyroid hormone (7).

Based on its etiology, HT is classified into two categories: Primary and secondary HT. Secondary HT is usually the result of drug-induced immune responses. Primary HT is the most common form of thyroiditis; it has a wide pathologic variety and includes six forms: Classic (8), juvenile (9) and immunoglobulin G4-dependent HT (10), the fibrous variant, Hashitoxicosis and painless thyroiditis (10,11), and its etiology has remained elusive. The common feature in all types of HT is lymphocyte infiltration and fibrosis (12,13).

Development of autoimmune destruction of thyroid cells and thyroid follicle atrophy is a multi-step process involving the convergence of genetic and environmental factors.

Environmental factors may act as triggers of autoimmune processes, including viral and bacterial infections, cytokine drugs, smoking, stress and pregnancy (14,15). Any cause of inflammation that may lead to the breakdown of tolerance can be considered as an environmental factor pre-disposing to the disease. However, it is clear that the type of immune responses is consistent with the function of numerous genes. These genes may be considered as susceptibility factors for autoimmune thyroiditis. The main candidates in this category are the genes human leukocyte antigen (HLA)-death receptor (DR)3, HLA-DR4, HLA-DR5, cytotoxic T-lymphocyte antigen-4 (CTLA-4), protein tyrosine phosphatase, non-receptor type 22, CD40, CD25, Fc Receptor Like 3 and forkhead box P3 (16,17).

CTLA-4 or CD152, a homo-dimer whose gene encodes 223 amino acids, is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. It is expressed on the surface

of activated T lymphocytes and has an important role in hemostasis and negative regulation of immune responses (18,19).

CTLA-4 has an inhibitory effect on immune responses through competition with CD28 co-stimulatory molecules in binding to B7-1 and B7-2 molecules on antigen-presenting cells. Balancing of CTLA-4/CD28 binding to their common ligand (B7 molecules) has a major role in determining the type of immune response. Therefore, it is obvious that factors regulating the expression or activation of CTLA-4 may affect this balance and a resulting loss of control of immune responses may lead to autoimmunity (20). Genetic variations may affect the expression and behavior of gene products. Numerous studies have shown the impact of the variations of the CTLA-4 gene on the function of its product, affecting the pathogenic pathways of autoimmune diseases. In the present study, the association of CTLA-4 gene polymorphisms [-318C/T (rs5742909) and +49A/G (rs231775)] with HT was examined.

Materials and methods

Patients. A total of 186 individuals who visited Aliasghar University Hospital (Zahedan, Iran), including 82 patients with HT and 104 age- and ethnicity-matched healthy controls, were recruited for the present study within 6 months from November 2015. Healthy controls had been referred for routine premarital blood tests. The patients' ethnicity was Sistani and Baloch (60 and 22, respectively), two ethnic populations of the Sistan and Balochestan province, who reside in Zahedan. HT was diagnosed based on diffuse goiter and clinical or biochemical features of hypothyroidism in the presence of autoimmune thyroiditis, i.e., positivity for anti-thyroid peroxidase or anti-thyroglobulin antibodies. All participants provided informed consent according to the Declaration of Helsinki and the study was approved by the ethics committee of Zahedan University of Medical Sciences (Zahedan, Iran).

Tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR). Genomic DNA was extracted from whole blood samples donated by each participant using a salting out protocol (21). The polymorphisms of the CTLA-4 gene, -318C/T (rs5742909) and +49A/G (rs231775), were genotyped using the T-ARMS-PCR technique. The sequences of inner and outer primers are listed in Table I, in addition to PCR product sizes and annealing temperatures. PCR products were evaluated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining (Fig. 1).

Statistical analysis. SPSS statistical software (version 18; SPSS, Inc., Chicago, IL, USA) was used for data analysis. The χ^2 test was applied to determine differences in genotypic and allelic distribution in the groups. $P<0.05$ was considered to indicate a statistically significant difference, while odds ratio (OR) and 95% confidence intervals (CIs) were also determined for estimation of differences. Deviation from the Hardy-Weinberg equilibrium was evaluated to examine the distribution of genotypes and alleles in patients and healthy controls.

Results

Patient characteristics. In the present study, 82 patients with HT were genotyped for the -318C/T (rs5742909) and +49A/G (rs231775) polymorphisms of the CTLA-4 gene. The mean age in the case group (91.5% females) was 39±11 years and that in the control group (70% females) was 37±12 years.

The -318C/T and +49A/G polymorphisms of the CTLA-4 gene are not associated with HT. The frequencies of genotypes and alleles are shown in Table II. In the cases and controls, the TT genotype was not found in the -318C/T polymorphism and the predominant genotype was CC, while the predominant genotype for the +49A/G single nucleotide polymorphism (SNP) was AA in both groups.

No significant differences in genotype and allele frequencies were observed between cases and controls regarding the -318C/T and +49A/G polymorphisms of the CTLA-4 gene. Genotype frequencies for -318C/T (rs5742909) and +49A/G (rs231775) polymorphisms were within the Hardy-Weinberg equilibrium ($P>0.05$).

The above calculations using gender-matched case and control groups did not result in statistically different genotype and allele frequencies of -318C/T and +49A/G polymorphisms of the CTLA-4 gene from those listed in Table II (data not shown).

Discussion

Taking into account the impact of CTLA-4 on immune system suppression, elements regulating its expression and function may be considered as important factors of immune system regulation. SNPs are considered normal variations of the human genome, and although they are known not to be direct causative factors of diseases, their effect on gene expression and its products renders SNPs important factors in disease susceptibility.

+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 of cell surface expression of CTLA-4 protein (22). The G allele has been associated with the reduction of T-cell proliferation (23). A meta-analysis study published in 2014 revealed that the CTLA-4 gene was associated with the risk of HT, and G allele carriers (GG + GA) of this polymorphism, considered as the dominant genetic model, were associated with an increased the risk of HT (24). However, in another meta-analysis study, this association was not identified (25).

The association between this polymorphism and other autoimmune diseases has also been investigated. In certain studies, the association of the GG genotype of +49A/G with autoimmune diseases was observed, including Type-I diabetes in a Kurdish Iranian population (26), rheumatoid arthritis in China (27) and celiac disease in Italy (28). However, in other studies, no correlation was identified between the +49A/G SNP of CTLA-4 and autoimmune diseases, including HT in Italy (29) and Lebanon (30), multiple sclerosis in Russia (31), common variable immune

Table I. Primers, annealing temperature and T-ARMS-PCR product sizes for cytotoxic T-lymphocyte antigen-4 polymorphisms including -318C/T (rs5742909) and +49A/G (rs231775).

SNP	Primer	Primer sequence	PCR product size (bp)	Annealing temperature
-318 C/T	318Fo	5'-CAATGAAATGAATTGGACTGGATG-3'	296	58˚C
	318Ro	5'-TGCACACACAGAAGGCTCTTGAATA-3'		
	318Fi(C)	5'-CTCCACTTAGTTATCCAGATCTTC-3'	C 201	
	318Ri(T)	5'-ACTGAAGCTTCATGTTCACTCTA-3'	T 141	
+49 A/G	49Fo	5'-GTGGGTTCAAACACATTTCAAAGCTTCAGG-3'	229	62˚C
	49Ro	5'-TCCATCTTCATGCTCCAAAAGTCTCACTC-3'		
	49Fi(G)	5'-GCACAAGGCTCAGCTGAACCTGGATG-3'	A 162	
	49Ri(A)	5'-ACAGGAGAGTGCAGGGCCAGGTCCTAGT-3'	G 120	

SNP, single nucleotide polymorphism; T-ARMS-PCR, tetra-primer amplification refractory mutation system polymerase chain reaction; Fo, forward outer; Ro, reverse outer; Fi, forward inner; Ri, reverse inner.

Table II. Genotype and allele frequencies of -318C/T (rs5742909) and +49A/G (rs231775).

SNP	Genotype/allele	Controls (n=104)	Patients (n=82)	Odds ratio (95% CI)	P-value	Statistical power
-318 C/T	TT, n (%)	0 (0)	0 (0)	-	-	
	TC, n (%)	8 (7.8)	9 (11)	1.5 (0.5-3.9)	0.1	4
	CC, n (%)	94 (92.2)	73 (89)	1	1	98
	T, n (%)	8 (4)	9 (5.5)		0.48	
	C, n (%)	196 (96)	155 (94.5)		-	
	AA, n (%)	57 (55.3)	50 (61)	1	1	
+49A/G	AG, n (%)	39 (37.9)	22 (26.8)	0.6 (0.3-1.2)	0.4	70
	GG, n (%)	7 (6.8)	10 (12.2)	1.2 (0.7-2.1)	0.4	11
	A, n (%)	153 (74)	122 (74)		0.98	
	G, n (%)	53 (26)	42 (26)			

CI, confidence interval; SNP, single nucleotide polymorphism.

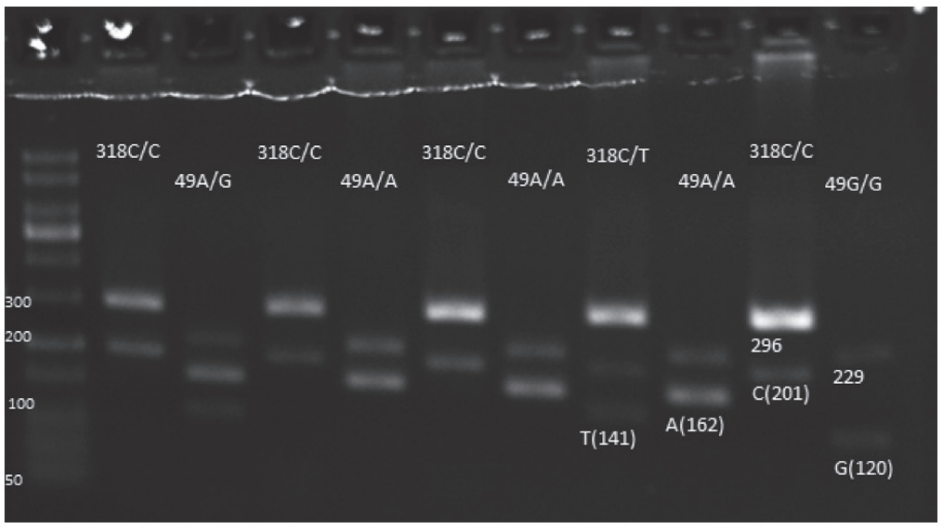

Figure 1. Agarose gel of the products of tetra-primer amplification refractory mutation system polymerase chain reaction for cytotoxic T-lymphocyte antigen-4 polymorphisms including -318C/T (rs5742909) and +49A/G (rs231775). The left-hand lane shows a marker.

deficiency in Caucasians (32) and autoimmune hepatitis in the Netherlands (33).

The present study revealed no association of any of the genotypes and alleles of the +49A/G (rs231775) polymorphism

in exon 1 of the CTLA-4 gene with HT in a cohort of 82 HT patients and 104 healthy individuals from Zahedan (Iran). Even in the dominant genetic model for G allele carriers (GG + GA), there was no significant difference between case and control groups (P=0.6).

It has been demonstrated that variations in the promoter region are likely to affect gene expression. The -318C/T (rs5742909) SNP is located in the promoter region of the CTLA-4 gene (34), and it has been suggested that this variation affects promoter activity and therefore the expression of CTLA-4 on the cell surface (35). Carriers of the T allele showed a marked enhancement of CTLA-4 mRNA expression and consequently, more expression of CTLA-4 protein on the cell surface (7). To date, only few studies have examined this variation and their results are contradictory: While certain studies have identified a correlation between the +49A/G SNP of CTLA-4 and autoimmune diseases [systemic sclerosis in Italy (36) and rheumatoid arthritis in Mexico (37)], others studies on other populations did not show any association of CTLA-4 SNPs with autoimmune thyroid diseases or other autoimmune conditions (38-41).

In the present study, no association between the -318C/T variation and HT was identified. Due to the fact that only few studies in this field of research have been performed on Iranian or even Middle Eastern populations, additional studies with a higher number of samples are recommended to identify whether the -318C/T (rs5742909) and +49A/G (rs231775) polymorphisms of CTLA-4 have any impact on HT.

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