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.

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 forhead 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

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

MEHRNAZ NAROOIE-NEJAD1,2, OMID TAJI3, DOR MOHAMMAD KORDI TAMANDANI1 and MAHMOUD ALI KAYKHAEI1,4

1Genetics of Non-Communicable Disease Research Center, Zahedan University of Medical Sciences, Zahedan, Sistan and Baluchestan 9816743463; 2Department of Genetics, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Sistan and Baluchestan 9816743463; 3Department of Biology, University of Sistan and Baluchestan, Zahedan, Sistan and Baluchestan 98155-987; 4Department of Internal Medicine, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Sistan and Baluchestan 9816743463, Iran

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Correspondence to: Dr Mahmoud Ali Kaykhaei, Department of Internal Medicine, School of Medicine, Zahedan University of Medical Sciences, Janat Boulevard, Hesabi Square, Zahedan, Sistan and Baluchestan 9816743463, Iran
E-mail: mazyar44@gmail.com

Key words: Hashimoto's thyroiditis, cytotoxic T-lymphocyte antigen-4, gene, polymorphism
of activated T lymphocytes and has an important role in hemo-
stasis 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 poly-
morphisms 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 electropho-
resis 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 χ² 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 frequen-
cies 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 expres-
sion 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 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-1
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
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

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).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>PCR product size (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>-318 C/T</td>
<td>318Fo</td>
<td>5'-CAATGAAATGAATTGGGACTGGATG-3'</td>
<td>296</td>
<td>58˚C</td>
</tr>
<tr>
<td></td>
<td>318Ro</td>
<td>5'-TGCAACACAGAAGGGTTCCTGAATA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>318Fi(C)</td>
<td>5'-CTCCACTTAGTTACGAGTCTTC-3'</td>
<td>C 201</td>
<td></td>
</tr>
<tr>
<td></td>
<td>318Ri(T)</td>
<td>5'-ACTGAAGCTTACAGTTGTCCTCTA-3'</td>
<td>T 141</td>
<td></td>
</tr>
<tr>
<td>+49 A/G</td>
<td>49Fo</td>
<td>5'-GTGGGTTCACACACATCTTAAAGCTTCAGG-3'</td>
<td>229</td>
<td>62˚C</td>
</tr>
<tr>
<td></td>
<td>49Ro</td>
<td>5'-TCTCATCTTCTGCTCTCAAGGTTCATCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>49Fi(G)</td>
<td>5'-GCACAAAGGCTCGAACCTGAGTG-3'</td>
<td>A 162</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49Ri(A)</td>
<td>5'-ACAGGAGAGGTCAGGGCCAGGTCCTAGT-3'</td>
<td>G 120</td>
<td></td>
</tr>
</tbody>
</table>

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).

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

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.
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.

References