ASSOCIATION OF FOXP3 GENE VARIANTS WITH RISK OF HASHIMOTO'S THYROIDITIS AND CORRELATION WITH ANTI-TPO ANTIBODY LEVELS

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Abstract

Context. Regulatory T cells (Tregs) have critical roles in preventing autoimmune diseases such as Hashimoto's thyroiditis (HT). Forkhead box P3 (Foxp3), the master transcription factor of Tregs, plays a pivotal role in Treg function.

Objective. Herein, we investigated the association of two single nucleotide polymorphisms (SNPs) of the Foxp3 gene with HT development.

Methods and study design. A total of 129 HT patients and 127 healthy subjects were genotyped for rs3761548 (-3279 A/C) and rs3761549 (-2383 C/T) in the Foxp3 gene, using polymerase chain reaction-restriction fragment length polymorphism.

Results. Genotypic and allelic distribution of rs3761548 SNP showed a significant association with HT. The CC genotype was observed in 37.2% of patients *versus* 22.1% of the controls [P<0.008, odds ratio (OR): 2.1; 95% confidence interval (CI): 1.2-3.6] and the AC genotype in 41.1% of patients compared to 54.3% of the controls (P<0.025, OR: 2.1; CI: 1.2-3.6). In addition, higher frequency of C allele in patients compared to controls (P=0.05, OR: 1.4; 95% CI: 0.9-2) suggested that patients with the CC genotype and C allele had increased susceptibility to HT. There were significantly higher serum levels of anti-thyroid peroxidase (ATPO) antibody in patients with the rs3761548 CC genotype (1156±163 IU/mL) compared to the other genotypes (≈582-656 IU/mL; P<0.004). We observed a greater frequency of the AC genotype in patients who had decreased ATPO antibody levels (P=0.02).

Conclusions. The association of the rs3761548 SNP with risk of HT and its influence on ATPO antibody levels suggested an important role for Foxp3 in the biology and pathogenesis of HT.

Key words: Hashimoto's thyroiditis, Regulatory T cell, Foxp3 gene, polymorphism.

INTRODUCTION

Hashimoto's thyroiditis (HT) is a chronic

inflammatory disease of the thyroid first documented over a century ago by Hakaru Hashimoto (1-3). Recent evidences have shown that genetic and environmental factors predispose people to HT (4, 5). HT is now considered the most prevalent organ-specific autoimmune disease, most common endocrine disorder, and most common cause of hypothyroidism (1, 3, 6-8). Hypothyroidism is the clinical hallmark of HT (9). The epidemiology of HT varies according to gender, age, geography, race, and iodine intake (2, 8-12). Due to the inaccurate diagnosis, the prevalence of HT in the different parts of the world is not fully explored. However, a recent report indicates that the prevalence in the general population is about 5% (9). The disease is diagnosed by clinical features, sonographic appearance of the thyroid, testing for thyroid-stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4) and presence of serum antibodies such as anti-thyroid peroxidase (ATPO) antibody, though a group of HT patients may be seronegative (6, 13). The immunopathogenesis of HT is still not fully understood. However, loss of immune tolerance and production of antibodies against thyroid antigens contribute to disease development (4). Over-activation of CD4+ T cells and their inflammatory cytokines produced by these cells may play a primary role in the pathogenesis of HT (2, 14). It is well known that regulatory T cells (Tregs) are responsible for the maintenance of self-tolerance (15). These cells play a key role in preventing autoimmune diseases by inhibiting auto-reactive T cells through two major mechanisms including cell-cell contact and secretion of suppressive cytokines. Failure or absence of Tregs contributes to autoimmune thyroid diseases (AITD) such as HT and Graves' disease (GD) (3, 15-21). Up-regulation of Tregs can suppress experimental autoimmune thyroiditis (4). Human Tregs are commonly identified by elevated expression of CD25, forkhead box P3 (Foxp3) positivity, and

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low expression/negativity for CD127 (15, 16). The transcription factor Foxp3 is the specific marker for human Tregs and considered to be the 'master regulator' of Treg differentiation and function (22-24). The occurrence of mutations and/or deficiency in the Foxp3 gene leads to a lack of Tregs or suppression of their regulatory function, which results in hyperactivation of autoreactive T cells and the consequent appearance of autoimmunity (24, 25). The Foxp3 gene is located in a region on chromosome Xp11.23 that has been shown to be linked with AITDs (4, 9, 25). Several single nucleotide polymorphisms (SNPs) in the promoter region of Foxp3 may affect the expression of this gene (4, 26). Recently, Bossowski *et al.* have demonstrated that the rs3761549 polymorphism in the Foxp3 gene could contribute to GD development in Caucasian female patients. However, there were no significant differences in rs3761547, rs3761548 and rs3761549 genotypes between HT patients and healthy controls (4).

To the best of our knowledge, there are no reports regarding the role of Foxp3 polymorphism in thyroid diseases from Iran. Therefore, in this study, we have intended to investigate the association of two polymorphisms, rs3761548 (-3279 A/C) and rs3761549 (-2383 C/T), in the FoxP3 gene with development of HT in Iranian patients. The relation of the genotypes to the levels of anti-thyroid peroxidase (ATPO) antibody as the main autoantibody generated in these patients was also investigated.

MATERIALS AND METHODS

Study subjects and sampling

A total of 129 Iranian patients diagnosed with HT (age: 38.1 ± 12.8 years) who referred from Shahid Mottahari Clinic, affiliated with Shiraz University of Medical Sciences, enrolled in this study. The control group consisted of 127 healthy subjects (age: 44.4 ± 2.2 years) who regularly donated their blood. All laboratory tests were normal and no signs of HT have been reported by the endocrinologist specialist. In addition, they did not have any family history of other autoimmune diseases. Patients were diagnosed by an endocrinologist according to the clinical diagnostic criteria indicative of hypothyroidism and laboratory findings such as increased in TSH with or without a decrease in thyroid hormone, T4 and the presence of the ATPO antibody. We did not take into consideration seronegative cases in this study. The Ethics committee of Shiraz University of Medical Sciences approved this study.

After we obtained consent from all participants, 6 mL peripheral blood was taken from each subject. The samples were then transferred to EDTA containing tubes for DNA extraction and clotting tubes for serum isolation.

DNA extraction

DNA extraction was performed using Genomic DNA Isolation Kit (from whole blood) (GeNet Bio, Korea) according to the manufacture's protocol. The quantity of DNA was determined using a spectrophotometer by measuring the ratio of absorbance at 260 and 280 nm, and the quality checked by running on the agarose gel. Extracted DNA was then stored at -20°C until use.

Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine Foxp3 gene rs3761548 and rs3761549 polymorphisms; PCR amplification for each SNP was performed using 1 μL of each forward and reverse primer, 10 μL master mix, 7.25 μL water and 0.75 μL genomic DNA. The sequences of forward (F) and reverse (R) primers for each SNP were obtained according to previous studies (27) as follows: For rs3761548: F: 5'-CCCTTGCTCTACTCCACGCCTCT-3', R: 5'-CAGCCTTCGCCAATACAGAGCC-3'. For rs3761549: F: 5'-CTGAGACTTTGGGACCGTAG-3', R: 5'-TGCGCCGGGCTTCATCGACA-3'. PCR conditions for the two SNPs were as follows. With respect to the rs3761548, the preheating was at 95°C for 5 min, denaturation at 95°C for 30s, annealing at 58.5°C for 45 s and extension at 72°C for 35 s. For rs3761549, preheating was at 95°C for 5 min, denaturation at 95°C for 30 s, annealing, 61°C for and 30 s and extension at 72°C for 35 s. A single final extension at 72°C for 10 min was used for both SNPs. The PCR program was repeated for 35 cycles. To verify the presence of PCR products, after completion of the reaction, 5 μL of the products were run on a 3% agarose gel. PCR product sizes for rs3761548 and 3761549 polymorphisms were 487 and 388 bp, respectively. Enzymatic digestion of DNA was carried out by using PstI enzyme for rs3761548 SNP and BsrI enzyme for rs3761549 SNP. The materials used for each sample were 0.66 μL buffer, 0.33 μL enzyme and 4 μL water. The PCR products were run on a 3% agarose gel to separate DNA fragments. Then, the bands were visualized under an ultraviolet transilluminator (Upland, CA). As shown in Figure 1, digestion of DNA by PstI enzyme resulted

Figure 1. Electrophoresis of PCR-RFLP products after enzymatic digestion of DNA by PstI for rs3761548 SNP (A) and by BsrI for rs3761549 SNP (B). The number of fragments resulting from the action of each enzyme, their size and the genotypes are shown. Molecular weight marker (Ladder) is 50 bp.

in two bands with sizes of 327 bp and 160 bp (CC) or three bands of 487 bp, 327 bp and 160 bp (AC). In AA genotype, DNA remained intact. DNA fragments produced by the action of BsrI enzyme were two bands of 261 bp and 127 bp (TT genotype) or three bands of 184 bp, 127 bp and 77 bp (CC genotype) or four bands of 261 bp, 184 bp, 127 bp and 77 bp (CT genotype).

Measurement of serum ATPO

After separating the serum from the blood, ATPO antibody was measured using enzyme-linked immunosorbent assay (ELISA) method (Bioassay Technology Laboratory, China). Serum levels > 40 IU/mL were considered as positive.

Statistical analysis

Genotypic and allelic differences between the patients and controls were analyzed by Chi-squared test. Odds ratio (OR) and confidence interval (CI) were also calculated using this test. For haplotype analysis, Alrequine software was used for 2 SNPs. With respect to the genotypes combination analysis the recessive and dominant model was used. ATPO antibody levels in different genotypes were compared with Kruskal-Wallis nonparametric test. $P < 0.05$ was considered as significant level.

RESULTS

Demographic characteristics of the patient and control groups

In this study, 129 patients with HT and 127 normal subjects were evaluated for Foxp3 gene polymorphism at the rs3761548 and rs3761549

positions. Females comprised the majority of individuals in the patient ($n=122$, 90.4%) and control ($n=120$, 92.9%) groups.

Genotypic and allelic distribution of Foxp3 gene (rs3761548 and rs3761549) polymorphisms in patients and controls

Foxp3 allelic and genotypic distribution in both patients and controls agreed with the Hardy–Weinberg equilibrium assumptions. Evaluation of the genotypic and allelic frequencies for the rs3761548 polymorphism (Table 1) showed that the AC carriers were more prevalent in both groups, though fewer patients had the AC genotype ($P=0.025$). The CC genotype accounted for 37.2% of patients and 22.1% of controls which indicated a significant difference between the patients and controls ($P=0.008$). The frequency of C allele was also significantly higher in patients (57.8%) than in controls (49.2%) ($P=0.05$). Genotype combination analysis showed a significantly higher frequency of the CC genotype compared to the AA+AC genotypes ($P=0.008$, OR: 2.1) in the patients compared to the controls. These data indicated that patients with the CC genotype and C allele had increased susceptibility to HT.

With respect to the rs3761549 polymorphism, Table 2 shows that the majority of patients (77.5%) and controls (81.1%) had the CC genotype. We observed that an approximately similar percentage of the patients and controls were carriers of C ($\approx 87\%$) and T ($\approx 13\%$) alleles. According to these data, there was no significant difference in rs3761549 genotypic and allelic distribution between the patients and controls.

Table 1. Frequency of different genotypes and alleles of the Foxp3 rs3761548 SNP in HT patients compared to healthy subjects

Genotype/allele	rs3761548 SNP			
	Patients	Controls	OR (95% CI)	P-value
AA	28 (21.7)	30 (23.6)	1.00 (reference)	-
AC	53 (41.1)	69(54.3)	0.58(0.3-0.96)	0.025
CC	48 (37.2)	28 (22.1)	2.1 (1.2-3.6)	0.008
A	109 (42.2)	129 (50.8)	1.00 (reference)	-
C	149 (57.8)	125 (49.2)	1.4 (0.9-2)	0.05
AA (R)	28 (21.7)	30 (23.6)	1.00 (reference)	-
AC+CC	101 (78.3)	97 (76.4)	0.8(0.5-1.6)	0.7
CC (D)	48 (37.2)	28 (22)	1.00 (reference)	-
AA+AC	81 (62.8)	99 (78)	2.1 (1.2-3.6)	0.008

Data are presented as number (%). R, recessive; D, dominant, Hashimoto's thyroiditis: HT; Forkhead box P3: Foxp3; SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval.

Table 2. Frequency of different genotypes and alleles of the Foxp3 rs3761549 SNP in HT patients compared to healthy subjects

Genotype/allele	rs3761549 SNP			
	Patients	Controls	OR (95% CI)	P-value
CC	100 (77.5)	103 (81.1)	1.00 (reference)	
CT	22 (17.1)	15 (11.8)	0.8 (0.4-1.4)	0.4
TT	7 (5.4)	9 (7.1)	1.62 (0.8-3.2)	0.2
C	223 (86.8)	221 (87)	1.00 (reference)	
T	34 (13.2)	33 (13.0)	1.02 (0.6-1.7)	0.9
CC (D)	100 (77.5)	103 (81.1)	1.00 (reference)	
CT+TT	29 (22.5)	24 (18.9)	0.6 (0.2-1.8)	0.4
TT (R)	6 (4.6)	9 (7.1)	1.00 (reference)	
CC+CT	123 (95.4)	118 (92.9)	1.62 (0.8-3.2)	0.2

Data are presented as number (%).R, recessive; D, dominant, Hashimoto's thyroiditis: HT; Forkhead box P3: Foxp3; SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval.

Haplotype analysis

The frequency of different haplotypes of two polymorphisms (AC, AT, CC, CT) with disease susceptibility did not reveal any statistically significant differences between the patients and controls. There was no significant data obtained when we analyzed the combination of genotypes from two SNPs (data not shown).

Relationship between rs3761548 and rs3761549 genotypes and ATPO antibody in patients

We investigated the possible correlation between genotypes and alleles of two selected SNPs in relation to the patients' ATPO antibody levels. The mean serum level of this antibody in patients was 818 ± 73 (standard error) IU/mL (median, 600 IU/mL; range, 63-5940 IU/mL). We observed that patients with the rs3761548CC genotype (1156 ± 163 IU/mL) had significantly higher serum levels of ATPO antibody than those with the AA (582 ± 89 IU/mL) and AC (656 ± 85 IU/mL) genotypes ($P=0.004$; Fig. 2). We classified the patients into three groups according to their ATPO antibody levels: low (less than 25th percentile, <243 IU/mL), moderate(25th-75th percentile, 243-1084 IU/mL), and high (more than 75th percentile, >1084 IU/mL). As

shown in Figure 3a, 59.4% of patients with high levels of ATPO antibody had the rs3761548 CC genotype and 56.3% of patients with low antibody levels had the AC genotype ($P=0.02$). The rs3761549 genotypes showed no significant difference in patients with different levels of ATPO antibody (Fig. 3b).

DISCUSSION

Tregs play a crucial role in maintaining immunological unresponsiveness to self-antigens and suppressing over-activation of immune responses to the host (25, 28). Foxp3 is an important transcription factor that has been widely studied over the past decade as a master regulator of Tregs. This transcription factor plays an essential role in the development and function of Tregs. Mutations in the Foxp3 gene cause a defective development of Tregs accompanied by autoimmune, inflammatory, and allergic diseases (4, 26, 29) The expression level of this transcription factor is an important factor in the regulation of immune homeostasis (4). Genetic defects in the Foxp3 gene cause immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, in which thyroid disease is a common complication (4, 30).

Figure 2. Anti-thyroid peroxidase (ATPO) antibody serum levels in the different genotypes of forkhead box P3 (Foxp3) rs3761548 and rs3761549 single nucleotide polymorphisms (SNPs) in Hashimoto's thyroiditis patients. Data are reported as the mean±standard error of the antibody levels. The rs3761548 CC genotype had significantly higher antibody levels compared to the other genotypes (*= $P<0.004$).

Figure 3. The percentage of patients with different forkhead box P3 (Foxp3) rs3761548 (a) and rs3761549 (b) genotypes according to percent of anti-thyroid peroxidase (anti-TPO) antibody. We divided the patients into three groups (low, moderate and high) of antibody levels and determined the percentage of each genotype in these groups. The rs3761548 CC genotype was significantly higher in the high anti-TPO antibody group, whereas the rs3761548 AC genotype was higher in the low anti-TPO antibody group ($P<0.02$).

Foxp3 promoter region polymorphisms can affect expression of this transcription factor (4, 26). Therefore, in the present study, we have evaluated the association of two Foxp3 rs3761548 and rs3761549 gene SNPs in patients with HT.

The results of the present study showed that more patients had the rs3761548 CC genotype than controls, which suggested that this genotype could predispose individuals to HT. It could be assumed that the increased frequency of the CC genotype might affect Foxp3 gene expression leading to defective Tregs and consequently increase the T cell response against thyroid gland antigens.

We found that unlike the CC genotype, fewer patients had the AC genotype of this SNP, which suggested a possible protective effect of this genotype for HT disease. Increased protection against disease development in AC heterozygous individuals for rs3761548 has been previously reported in a study in Egyptian patients with psoriasis (31). Evaluation of the frequency of rs3761548 A and C alleles in the patients and controls showed a significant elevation of the C allele in patients, which was in line with the higher genotypic CC distribution in this group. We also studied the frequency of genotypes and alleles in the rs3761549 SNP; however, we did not find any significant difference in the frequency of genotypes and alleles of this SNP, which suggested that this polymorphism had no association with HT susceptibility. The frequency of haplotypes and the combined genotypes of the rs3761548 and rs3761549 SNPs was also compared in the patient and control groups and we did not observe any significant difference.

Few studies have assessed Foxp3 polymorphism in patients with autoimmune thyroiditis, especially in HT patients (4, 26, 32-35). According to these studies, it has been suggested that decreased Foxp3 gene expression due to functional polymorphisms could predispose individuals to AITD. However, other studies did not confirm this finding. Owen *et al.* reported no evidence that Foxp3 gene polymorphisms (including rs3761549) contributed to GD susceptibility in the UK population (36). In another study, there was no reported association of rs3761548 and rs3761549 polymorphisms with development of GD and HT (33). These findings contradicted a study by Bossowski *et al.*, in which the researchers observed a significant difference in the rs3761549 polymorphism between GD patients and healthy controls. The findings by Bossowski *et al.* were similar to the current study where we did not

observe this difference in HT patients (4). They also found no difference in the frequencies of rs3761548 variants between HT patients and controls, which contrasted the current study findings of an association of rs3761548 polymorphism with HT development. These differences might be attributed to the differences in ethnic backgrounds of the studied populations.

ATPO antibodies are the most common antibodies in patients with HT. The level of this antibody is proposed to be a reflection of disease activity. Karanikas and colleagues have shown a correlation between the ATPO antibody level and parameters of disease activity that include increased production of cytokines that enhance CD4+ and CD8+ T cells function, such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α (37). Moreover, ATPO antibodies may act cytotoxically on thyrocytes suggesting their role in the deterioration of thyroid function (35, 38).

We investigated the serum ATPO levels in different genotypes of two selective polymorphisms in HT patients. Interestingly, the results indicated that patients with the rs3761548 CC genotype had significantly higher levels of autoantibody compared to AA and AC genotypes. Investigators proposed that ATPO antibody levels might have a correlation with disease activity; therefore, in addition to increased susceptibility to HT, the CC polymorphism might have a relation to HT disease activity.

We categorized patients into three groups according to their sera levels of this antibody – low, moderate, and high – in order to find the percentages of each genotype in these groups. We found a higher number of patients with the rs3761548 CC genotype in the high group and a higher number of patients with the AC genotype in the low group. These results, in addition to confirm a positive relationship between the CC genotype and ATPO levels, may suggest a higher frequency of the AC genotype in patients with decreased disease activity. This finding was in line with a previously mentioned protective role of this genotype in HT disease. A previous study evaluated the rs3761548 and rs3761549 polymorphisms in a Japanese population of patients with both severe and mild forms of HT and GD. Their study showed a direct relationship between the presence of the Foxp3 gene rs3761549 polymorphism and the severity of HT (33), and suggested an impact of Foxp3 polymorphisms on disease prognosis.

The current study has some limitations. We did not determined the severity of HT in patients according

to clinical features; thus, further evidence would be needed to confirm the relation of genetic variants with patients' clinical features. Studies with a larger number of subjects might be of benefit to verify our results.

In conclusion, we found an association of genotypic and allelic frequency of rs3761548 but not rs3761549 polymorphism with HT. There was a significant correlation between the level of ATPO antibody and the frequency of rs3761548 CC genotype which may show a possible association of this genotype with disease activity in addition to its relation to disease development. Finding the relationship between Foxp3 gene polymorphisms and development of HT can provide a better understanding of immune regulatory mechanisms, pathogenesis, and prognosis in this disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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