Association between TPO Asn698Thr and Thr725Pro gene polymorphisms and serum anti-TPO levels in Iranian patients with subclinical hypothyroidism

Amirhosein Khoshi,1 Alireza Sirghani,2 Mehran Ghazisaeedi,3 Ali Zarei Mahmudabadi,2 Amir Azimian4

1Department of Medical Biotechnology and Molecular Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, IR; 2Department of Biochemistry, Baqiyatallah University of Medical Sciences, Tehran, IR; 3Department of Internal Medicine, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, IR; 4Department of Pathobiology and Laboratory Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, IR Iran

ABSTRACT

OBJECTIVE: Subclinical hypothyroidism (SCH) is defined as high levels of TSH in the presence of normal levels of serum FT4. Since thyroid peroxidase (TPO) plays a key role in thyroid hormone synthesis, variations in the TPO gene can change the enzyme structure and result in the production of anti-TPO antibodies. The aim of this study was to examine the relationship between the Asn698Thr (A2095C) and Thr725Pro (A2173C) polymorphisms of the TPO gene and anti-TPO levels in patients with SCH. DESIGN: In this study, 150 individuals (75 cases and 75 controls), aged 19-75 years, were selected randomly by a clinician. The thyroid function tests included were FT3, FT4, TSH and anti-TPO antibodies using ELISA. The TPO gene polymorphisms were examined by PCR-RFLP. RESULTS: Anti-TPO levels in the experimental group was significantly increased (P=0.020). The A2095C genotype frequency in the experimental and control groups were 37.3% vs 34.7% for the AA healthy genotype, 20% vs 46.7% for AC and 42.7% vs 18.6% for CC, respectively (P=0.001). The A2173C genotype frequency in the experimental and control groups were 22.6% vs 68% for healthy AA, 40% vs 25.3% for AC and 37.4% vs 6.7% for CC, respectively (P<0.001). The increased anti-TPO antibodies were significantly associated with the A2173C polymorphism (P=0.035). The findings showed that the chance (odds ratio) of developing subclinical hypothyroidism in individuals who had C alleles was 1.5 and 5.6-fold higher than in individuals without these alleles in the A2095C and A2173C regions, respectively. CONCLUSIONS: Determination of anti-TPO antibody levels and exon 12 TPO gene polymorphisms in patients with SCH can be helpful for prediction of overt hypothyroidism.

Key words: Anti-TPO, Gene polymorphism, Subclinical hypothyroidism, Thyroid peroxidase
INTRODUCTION

Hypothyroidism may occur due to congenital thyroid abnormalities, iodine deficiency and autoimmune thyroid disease/Hashimoto’s thyroiditis (AITD/HT) in which antibodies against thyroid peroxidase (anti-TPO) serve as a clinical marker for the early detection of AITD/HT.1,2 Subclinical hypothyroidism (SCH) is defined as high serum thyrotropin or thyroid stimulating hormone (TSH) and normal levels of free thyroxine (FT4).3 Subclinical hypothyroidism is mild thyroid failure: it is a common condition with a prevalence of 3% to 8% in the population without known thyroid disease. The prevalence of SCH increases with age and is higher in women.4,5

Since serum TSH has a log-linear association with circulating thyroid hormone levels, measurement of TSH is the necessary test for diagnosis of mild thyroid failure when the peripheral thyroid hormone levels are within the normal laboratory range.3 About 80% of patients with SCH have a serum TSH of less than 10 mIU/L, usually between 5-10 mIU/L, as is typical of most SCH cases.6 It is estimated that patients with SCH have a high rate of progression toward clinically and also biochemically overt hypothyroidism, 2.6% each year if anti-TPO antibodies are absent and 4.3% if they are present. However, some persons do not show progression, while some experience normalization.6,7

Thyroid peroxidase (TPO), a glycoprotein with enzyme activity in thyroid metabolism, plays a critical role in thyroid hormone synthesis, including thyroxin (T4) and 3-3’-5-triiodothyronine (T3), which have important roles in growth control, differentiation, regulation of metabolism and the physiological functioning of virtually all human tissues.8,9

The TPO gene on the short arm of chromosome 2, locus 2p25, consists of 17 exons.8,10 Some studies have suggested that mutations in the TPO gene with an autosomal recessive inheritance pattern are common causes of autoimmune thyroid diseases11,12 because some amino acid substitutions in the TPO protein may lead to recognition of this enzyme as a foreign antigen by the immune system and anti-TPO autoantibodies can be secreted against its actions.13,14

The prevalence of genetic single nucleotide polymorphisms (SNPs) of the TPO gene differ according to race and/or between different populations, while TPO gene polymorphisms have been implicated in the pathogenic mechanism of hypothyroidism.13,16 One of the most common SNPs of the TPO gene which is reported in the Iranian population was identified in exon 12, this being related to increased levels of anti-TPO antibodies in patients with hypothyroidism.16

Previous studies have indicated that TPO genetic polymorphisms may be associated with serum levels of anti-TPO antibodies.16-19 In addition, anti-TPO levels in 80% of patients with SCH are higher than in normal individuals.6

Based on the importance of preventing the manifestation of overt hypothyroidism and the high levels of serum anti-TPO antibodies that occur in most patients with SCH, the aim of this study was to investigate the frequencies of some genetic polymorphisms of the TPO gene on exon 12, including A2095C (rs121908087) and A2173C (rs732609), which are responsible for amino acid substitution in the TPO protein at 698 Asn→Thr and 725 Thr→Pro, respectively; and compare these polymorphisms with anti-TPO levels in Iranian patients with subclinical hypothyroidism so as to explore the association between these polymorphic regions and anti-TPO antibody levels.

MATERIALS AND METHODS

Study population

A case-control study was performed in the city of Bojnurd in Northeast Iran between September 2015 and June 2016. Informed consent was obtained from each individual included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution’s human research committee of the North Khorasan University of Medical Sciences (ethic code: IR.nkums.REC.1394.142).

Eligible participants were 150 unrelated individuals including 75 cases with subclinical hypothyroidism and 75 healthy individuals without any complications as the control group, who were matched according to age (19-75y) and sex, without a history of thyroid dysfunction, who were admitted randomly to enroll in the present study and referred to the clinical labo-
ratory of the Imam Reza Hospital of Bojnurd. Care was taken to ensure that the participants' mean ages in the two study groups were comparable.

On the basis of clinical and laboratory investigations by a physician, 75 patients were selected as cases with subclinical hypothyroidism who had normal Free T3 and Free T4 levels and TSH levels higher than 5.2 mIU/L, while 75 individuals with normal levels of Free T3, Free T4 and TSH were designated as the control group.

The structured questionnaire included age, sex, smoking habits, family history of thyroid diseases, history of neck surgery, history of drug usage, especially antidiabetic, lipid-lowering and antihypertensive drugs.

Patients with diabetes mellitus and/or with any liver, cardiovascular, rheumatologic, renal or thyroid diseases and also participants who had taken antihypertensive, antidiabetic, lipid-lowering and antithyroid drugs during 1 month before sampling were excluded from the study.

**Laboratory and molecular diagnosis**

The Free T3, Free T4, TSH and thyroid peroxidase antibody levels were measured via the Enzyme-linked immune absorbent assay (ELISA) method (Monobind, USA) using a plate reader (BioTek, USA).

For analysis of TPO gene polymorphisms, blood samples were collected in EDTA-coated tubes and stored at -20 °C until genomic DNA was extracted using the Proteinase K column method according to the protocol of the manufacturer (Sinaclon, Iran). After extraction, all DNA samples were measured by UV spectrophotometer and their purity was checked by A260/A280 ratio.

**TPO** A2095C (Asn 698 Thr) and A2173C (Thr 725 Pro) gene polymorphisms were determined by polymerase chain reaction (PCR) using premix tubes (Sinaclon, Iran) and followed by restriction fragment length polymorphism analysis (RFLP).

The PCR was used to amplify 209 bp for A2095C and 131 bp fragments for the A2173C polymorphism SNP in exon 12 of the TPO gene using the following oligonucleotide primers:

F: 5’-GACTTGGAAGGCATCCATGG -3’ for A2095C and F: 5’- TGCCCATGGATGCTTCCAAG -3’ and R: 5’- GCTCTCTGGGAAGCCACACT -3’ for A2173C polymorphisms.

Polymerase chain reactions were carried out in a Veriti thermal cycler (Applied Biosystems, USA) in which DNA templates were denatured at 95°C for 5 minutes, amplification, consisting of 40 cycles at 95°C for 45 seconds, 58°C for 1 minute and 72°C for 45 seconds, with a final extension at 72°C for 5 minutes.

After that, the PCR products were subjected to restriction enzyme analysis by digestion at 65°C for 8 h with BseNI (BsrI) restriction endonuclease (ThermoFisher Scientific, USA). In the RFLP tests, each restriction endonuclease mixture (total volume 30 ul) contained 15 ul amplified fragments, 12 ul DNase free distilled water, 2 ul appropriate buffer and 1 ul of BseNI restriction endonuclease (1 unit of restriction enzyme is the amount of enzyme required to digest 1 ug of lambda DNA in 1 h at 65 °C for BseNI). Restriction products were separated by electrophoresis on a 3% agarose gel in TBE buffer and stained with DNA safe stain.

For confirmation of A2095C and A2173C SNPs in exon 12 of the TPO gene, the PCR products were investigated by sequencing and analyzed by MEGA 4.1 software.

**Statistical analyses**

SPSS software (version 20) was used for statistical analysis. Comparisons of demographic and thyroid panel tests between study groups were analyzed using the paired t-test. All data are expressed as mean±SD. The frequencies of alleles and genotypes between the experimental and control groups were analyzed using the Chi-squared test. In addition, the correlation between genotype alleles with anti-TPO levels was examined by the Pearson correlation test. P values lower than 0.05 were considered statistically significant.

**RESULTS**

General characteristics and the thyroid function profile of participants are presented in Table 1. TSH means in the experimental and control groups were 8.8±0.36 and 3.1±0.12, respectively (P <0.001), but
in the FT3 and FT4 levels there were no significant differences between study groups. In addition, anti-TPO levels in patients with subclinical hypothyroidism were higher than those in the control group ($P = 0.020$). The power calculation of this study was estimated at around 80%.

**TPO A2095C and A2173C genotypes determination**

As per the Hardy-Weinberg Equilibrium (HWE) law, there are two alleles of the *TPO* gene. For determination of the A2095C polymorphism, digested fragments of 92, 79 and 38 bp were detected in the healthy homozygotes for the A allele (genotype AA), while the digested fragments 117, 92, 79 and 38 bp were detected in the heterozygotes (genotype AC) and the digested fragments 117 and 92 bp were detected in the homozygotes of the C allele (Figure 1). In order to identify the A2173C polymorphism, the undigested fragment of 131 bp was detected in healthy homozygotes for the A allele (genotype AA), the digested fragments 68 and 63 bp were detected in homozygotes for the C allele (genotype CC) and both digested and undigested fragments 131, 69 and 62 bp were detected in heterozygotes (genotype AC) (Figure 2).

Table 2 shows the *TPO* A2095C and A2173C genotype frequencies in the experimental and control groups. Frequencies of heterozygosity for the A/C allele 2095 in the experimental and control groups were 20% and 46.7%, respectively; however, the frequencies of the two allele polymorphisms (CC) in the experimental and control groups were 42.7% and 18.6%, respectively ($P = 0.001$) (Figure 3). Furthermore, the
TPO gene polymorphisms and subclinical hypothyroidism

Whenever there was an association between SNPs and anti-TPO antibody levels in the study groups there were no significant differences among serum anti-TPO levels and the three genotypes, namely AA, AC, and CC in the A2095C polymorphism (P = 0.086), while there were significant differences between anti-TPO levels and the three genotypes, namely AA, AC and CC in the A2173C polymorphism region of the TPO gene (P = 0.035) (Table 3); these data regarding each SNP were calculated in the whole of the study groups. However, there was no significant correlation between other thyroid function tests in the genotypes of the TPO A2095C and A2173C polymorphic regions. The genotypes and allele frequencies of the A2095C and A2173C polymorphisms in the whole of the study population who had positive and negative anti-TPO antibody levels are depicted in Table 3.

The findings of the present study demonstrate that there is a significant correlation between the C allele of both the TPO A2095C and A2173C gene polymorphisms and patients with subclinical hypothyroidism (P = 0.001 and P <0.001, respectively). Investigation of the A2095C and A2173C polymorphisms in the experimental group revealed that the odds ratio (CI 95%) in one or two alleles is 1.5 and 5.6, respectively. Indeed, the probability ratio of the risk of subclinical hypothyroidism in patients who have C alleles in the A2095C polymorphic region is 1.5, but in the A2173C region it is 5.6-fold higher than those without this allele. For example, if someone has the A2173C polymorphism (i.e. AC or CC), the likelihood of his having SCH will be 5.6 times higher than in an individual who does not have this polymorphism. In addition, there is a significant association between high serum anti-TPO levels and subclinical hypothyroidism, this being substantiated

Table 2. Comparison of TPO Asn698Thr (A2095C) and Thr725Pro (A2173C) genotype frequencies in study groups

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<th>A2095C polymorphism</th>
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<th>A2173C polymorphism</th>
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<tr>
<td>Case</td>
<td>SCH</td>
<td>37.3%</td>
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<td>Control</td>
<td>Normal</td>
<td>34.7%</td>
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Figure 3. Frequencies of Asn 698 Thr (A2095C) polymorphism in study groups. A2095C genotypes include AA (normal), AC (polymorphism in one allele), CC (polymorphism in two allele).

Figure 4. Frequencies of Thr 725 Pro (A2173C) polymorphism in case and control groups. A2173C genotypes include AA (normal), AC (polymorphism in one allele), CC (polymorphism in two allele).
in our study by the fact that 45.3% of the experimental individuals had elevated anti-TPO antibodies (P = 0.015). As expected, the odds ratio test showed that the risk of subclinical hypothyroidism in individuals with elevated serum anti-TPO was 19.9 times higher than in those with negative anti-TPO. According to the results of the Pearson correlation test, there was a significant relationship between anti-TPO and TSH levels in the case and control groups (P = 0.001 and P = 0.006, respectively).

**DISCUSSION**

The present study provides evidence of an association between A2095C (rs121908087) and A2173C (rs732609) genetic variations in the TPO gene with anti-TPO levels in patients who have subclinical hypothyroidism (SCH). The results showed that these polymorphic regions have a significant correlation with subclinical hypothyroidism. In addition, as expected, the serum anti-TPO titer had increased significantly in the patient group, especially in those who have the C allele in the A2173C polymorphic region. Furthermore, we detected the presence of amino acid substitutions, such as Asn698Thr for A2095C and Thr725Pro for the A2173C polymorphism, on performing bioinformatics studies.

Autoimmune thyroid disease (AITD) is thought to arise from a combination of genetic and environmental factors.\textsuperscript{17} Since the protein structure of thyroid peroxidase is related to genetic variations in the TPO gene, TPO appears to play a critical role in thyroid hormone synthesis; however, genetic variations may lead to changes in its structure, thus the TPO enzyme is recognized as a foreign body and antibodies are produced against it.\textsuperscript{1} Hashimoto’s thyroiditis (HT), an autoimmune disease in which the thyroid gland is gradually destroyed by lymphocyte invasion and antibody-mediated immune processes, is the most common cause of hypothyroidism\textsuperscript{6,17} and may occur as a result of subclinical hypothyroidism.\textsuperscript{16}

Since the TPO enzyme structure and/or its activity is defective in autoimmune thyroid disease, for the first time the hypothesis is being proposed that genetic variations of the TPO gene may contribute to the occurrence of these diseases.\textsuperscript{13,14}

Based on the results of the present study, in the presence of the C allele of A2173C SNP, anti-TPO antibody levels would increase in patients with subclinical hypothyroidism; however, we did not observe any correlation between increased anti-TPO levels and the C allele of A2095C.

Because of the high prevalence of subclinical hypothyroidism and its associated metabolic risk factors such as hyperlipidemia, the American Thyroid Association recommends screening tests by measurement of serum TSH after the age of 35 at 5-year intervals.\textsuperscript{20}

To date, several studies have found evidence of the association of TPO gene polymorphisms with anti-TPO levels in patients with hypothyroidism; however, the genetic variations in the TPO gene and their correlation with anti-TPO antibodies levels have not been investigated in patients with subclinical hypothyroidism who do not have overt hypothyroidism and its symptoms.

In line with studies which indicated that TPO gene variations such as polymorphisms are associated with anti-TPO levels\textsuperscript{17,19}, as well as with some studies in patients with overt hypothyroidism in the Tehran population,\textsuperscript{1,16} the present study also indicates the association of genetic characterization of the TPO gene with anti-TPO levels. However, the novelty of our study was to investigate exon 12 TPO gene polymorphisms in patients with subclinical hypothyroidism.
thyroidism who do not have overt hypothyroidism; furthermore, we have demonstrated the relationship between the Asn698Thr polymorphism and subclinical hypothyroidism for the first time.

Schultheiss et al. have studied subjects of Caucasian ancestry in three independent prospective populations including the Atherosclerosis Risk In Communities Study, the Study of Health in Pomerania and the Study of Health in Pomerania (SHIP-TREND). They have identified four novel genetic loci and confirmed five known loci associated with anti-TPO antibody levels. A Genetic Risk Score (GRS) based on index SNPs in these nine loci (including RERE, MAGI3, TPO, KALRN, HCP5, HLA-DOB, HLA-DPB1, BACH2, ATXN2) revealed strong and graded associations not only with anti-TPO levels and positivity but also with TSH and FT4 concentrations (P <0.001), as well as with the clinical entities subclinical and overt hypothyroidism and goiter. Furthermore, they have shown that in comparison with individuals in the lowest GRS quartile, those in the highest quartile had 1.80-fold higher odds of subclinical hypothyroidism and 1.89-fold higher odds of overt hypothyroidism.17 This study points to the importance of the nine genetic variations, among them in particular the TPO gene SNP rs11675434, in patients with autoimmune hypothyroidism.

Faam et al have reported that TPO genetic variations of exon 11 and exon 12, the polymorphisms of T1936C and A2257C, but not of T2229C, are significantly associated with high levels of serum anti-TPO antibodies in patients with hypothyroidism; however, these associations were attenuated after adjustment for sex and age.1 In the present study, there was no relationship between patient’s age and either TPO A2095C or A2173C polymorphisms. Our Primer-BLAST investigation revealed that some electrophoretic bands and primer sequences in the study of Faam et al.1 were not matched to the TPO gene, thus we cannot cite and compare our findings with this study.

Bikker et al reported that a genetic variation in exon 10 of the TPO gene has a significant effect on the enzyme activity of TPO.21 Brčić et al. have reported that two SNPs (rs1077462, rs11675434) are associated with anti-TPO antibodies levels in patients with Hashimoto’s thyroiditis. They suggested that genetic variations in or near the TPO gene are associated with HT.3 Balmiki et al. have demonstrated that two TPO gene polymorphisms, namely Thr725Pro (rs732609) and Asp666Asp (rs1126797), had a significant correlation with those of Indian patients with hypothyroidism.15 Similarly, we were also able to show the significant relationship between the Thr725Pro (A2173C) gene polymorphism of TPO and patients who had elevated serum TSH, but not FT3 and FT4, who were identified as having subclinical hypothyroidism. Hedayati et al. have investigated the effect of two polymorphisms of the TPO gene, namely T1193G in exon 8 and T2145C in exon 12, and their correlation with anti-TPO serum levels in Iranian patients with hypothyroidism.16 They reported that T1193G was not polymorphic and that there was no association between this SNP and serum anti-TPO levels, whereas the presence of the C allele in the T2145C polymorphism was correlated with increased levels of serum anti-TPO antibodies and was also significantly associated with serum anti-Tg levels,16 this confirmed by the fact that 71.2% of hypothyroid patients had the C allele in this polymorphic region. In addition, the frequency of the C allele, particularly of the CC genotype, was more closely related to serum anti-TPO levels (OR: 9.2). In the present study, the C allele of both A2095C and A2173C polymorphic regions, especially of the CC genotype, was also associated with patients with subclinical hypothyroidism (OR: 1.5 and 5.6 for A2095C and A2173C, respectively). It is necessary to note that we could not find the T2145C polymorphism as reported in the study by Hedayati et al16 given that their results had an explicit bias, particularly in primer design and the use of the BsrI enzyme digestion site, hence the sequence in the 2145 nucleotide position is CCCCG, which cannot be recognized by this restriction enzyme. BseNI (BsrI) can recognize the ACTGGN sequence and digest the PCR product at the A↓ position; this digestion type was observed in the A2173C and A2095C polymorphic regions. Indeed, both polymorphic regions were changed to C according to our sequencing data.

Our study had some potential limitations. First, we acknowledge that the sample size of the present study was relatively small and that the frequency of
TPO genetic polymorphisms should be confirmed by investigation of a larger population. In addition, most of the patients diagnosed as SCH were female. Furthermore, in concordance with previous studies which have focused on exon 12 of the TPO gene, we chose two candidate gene polymorphisms that can create missense mutations; certainly, additional studies on more polymorphisms of the TPO gene will provide further insights.

In summary, the findings of the present study demonstrate that 45.3% of patients with subclinical hypothyroidism had positive anti-TPO antibodies, while our statistical analyses have shown that the probability risk of disease in individuals with positive anti-TPO antibodies is 19.9 times higher than those with normal levels of serum anti-TPO. Since there is a correlation between the C allele of the A2173C polymorphism and anti-TPO levels, it could be hypothesized that the presence of the C allele at 2173 nucleotide in exon 12 of the TPO gene could have an important role in the change of the thyroid peroxidase protein structure, thus it may produce autoantibodies against this protein. Therefore, on the basis of the demonstrated association of TPO gene polymorphisms with racial differences in the incidence of autoimmune thyroiditis, we suggest that the evaluation of the gene polymorphisms of TPO exon 12 and the measurement of anti-TPO antibodies levels is likely to be helpful in Iranian patients with subclinical hypothyroidism and that, with time, therapeutic intervention may be able to prevent the incidence of overt hypothyroidism in these patients.

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DISCLOSURE STATEMENT

The authors have nothing to disclose.

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