ASSESSMENT OF THE ROLE OF THYROID PEROXIDASE GENE POLYMORPHISM AS A CAUSE OF HYPOTHYROIDISM IN ZAGAZIG UNIVERSITY HOSPITAL PATIENTS

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ABSTRACT

Background: Thyroid peroxidase (TPO) is the main enzyme involving the thyroid hormone biosynthesis. Thus studying its gene mutation explains some of etiological factors of chronic autoimmune hypothyroidism. The changes in enzyme built structure due to gene polymorphism may stimulate autoantibody production that may cause thyroid destruction. Thus, we correlate the TPO gene mutation with anti TPO-ab serum levels.

Subjects and Methods: 25 participants from Zagazig University hospital were recruited in patient group with elevated serum TSH, low serum fT4 and positive anti TPO-ab level and history of hypothyroidism. 25 healthy subjects were recruited in control group. Two genetic variants in TPO gene -Thr^{725}Pro of exon 12 and Arg^{386}His of exon 8- were genotyped in both groups using polymerase chain reaction–restriction fragment length polymorphism technique. BsaJI and SacII restriction enzymes were used on the PCR products, respectively.

Results: The carriage of Pro allele (in Thr^{725}Pro polymorphism of exon 12 of TPO gene), probably increased the susceptibility of autoimmune hypothyroidism (OR = 2.471, 95% CI = 1.100-5.547, and P = 0.027). Also, the carriage of Pro allele significantly increase the anti TPO-ab serum levels (P<0.0001). On the other hand, patients with His allele (in Arg^{386}His in exon 8 of TPO gene), showed lower susceptibility to autoimmune hypothyroidism with no significant association with anti TPO-ab levels.

Conclusion: This study found an association of Thr^{725}Pro of exon 12 of TPO gene polymorphism with autoimmune hypothyroidism in Egyptians and correlated the anti-TPO-ab levels with different genotypes in hypothyroid patients. Also, it found no association of Arg^{386}His of exon 8 of TPO gene with autoimmune hypothyroidism, and no relation was found between the anti TPO-ab levels in hypothyroid patients and its different genotypes in Egyptian population.

Key words: Autoimmune – Hypothyroidism - Hashimoto's disease - Anti-TPO - Gene polymorphism - TPO gene - Thr^{725}Pro -Arg^{386}His

INTRODUCTION

Disturbance of thyroid hormone synthesis and its actions are one of the most common endocrine distresses.[1] Endemic goiter is the main type of primary hypothyroidism in iodine deficiency areas. However, in countries with adequate dietary iodine, hypothyroidism is chiefly caused by thyroiditis.[2] thyroiditis results from preceding or presently current inflammation of the thyroid gland and presents in different ways. The commonest thyroiditis form is chronic autoimmune thyroiditis (also called; Hashimoto thyroiditis).[3] This type of thyroiditis affects about 2 to 4% of women and up to 1% of men worldwide and the prevalence rate increases with advancing age.[4]

Studies concentrated on chronic autoimmune hypothyroidism risk factors have reported that thyroid peroxidase (TPO) gene mutation is the most important gene mutation affecting the thyroid hormone synthesis.[5],[6]

TPO is also called iodide:hydrogen-peroxide oxidoreductase.[7] It is the key enzyme in the thyroid hormone biosynthesis. It catalyzes tyrosil residues iodination and coupling with thymoglobulin (Tg).[8] TPO enzyme is a dimer membrane-bound glycoprotein. Each monomer consists of 933 amino acid residues.[9] It is formed from extracellular part that formed mainly from a peroxidase domain, a transmembrane helix, and a short C-terminal intracellular tail.[10] The human TPO gene ID: 7173 is located on chromosome 2p25 and spans approximately 150 Kb, containing 17 exons and 16 introns.[11] In Hashimoto's disease, the thyroid gland is steadily damaged by a multiple types of cells and antibody mediated immune processes.[12] Anti-TPO-antibodies (anti-TPO-ab), thyroglobulin antibodies and thyrotropin receptor antibodies are the main clinically important anti-thyroid autoantibodies. Although there is overlap, anti-TPO-ab represents the most important auto-antigen in autoimmune thyroid diseases.[13] The changes in enzyme built structure can evokes autoantibodies that are markers of thyroid infiltration or implicated in thyroid destruction.[14],[15],[16]

The anti-TPO-ab presents in approximately 90% of HT [17],and its high levels are associated with active phase of autoimmune thyroid disease. Therefore, an antibody assay is essential to evaluate illness severity within patients. [17], [14]

This study tried to detect the genetic etiology of hypothyroidism by association of the disease with two different gene polymorphisms: $Arg^{386}His$ of exon 8 and $Thr^{725}Pro$ of exon 12. Also, we associated the elevation of anti TPO-ab serum level with the studied polymorphisms. This study aimed to improve early detection and outcome of hypothyroid disease and aid in developing a genetic screening protocol.

SUBJECTS AND METHODS

This study was carried out in the Molecular Biology Laboratory in the Medical Biochemistry and Endocrinology Unit of Internal Medicine Departments, Faculty of Medicine, Zagazig University. This case-control study included 25 subjects with hypothyroidism with positive anti-TPO-ab in patient group, and 25 subjects with no history of hypothyroidism and negative anti-TPO-ab in control group. The design of this study was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. History collection was done to all study participants. They were subjected to complete systemic examination and clinical evaluation of thyroid gland enlargement (goiter, if present). Obesity, cold intolerance, lethargy, muscle cramps and constipation were evaluated according to hypothyroid assessment roles of **Zulewski et al.**[18]

Blood sampling: 4 ml of venous blood was withdrawn from each subject and divided to 2 portions: one ml in tubes containing ethylenediaminetetraacetic acid, dipsodium salt, dihydrate ($Na_2EDTA\text{-}2H_2O$), for genomic DNA extraction. Serum was separated from remaining three ml for ELISA procedures. Collected serum and EDTA-sample were stored at -20 ^oC till use.

Estimation of serum levels of thyroid-stimulating hormone (TSH): Serum levels of TSH were measured using Thyroid-stimulating hormone enzyme immunoassay kit (Chemux Bioscience, Inc.) following manufacturer's instruction. The TSH assay procedure depends on the rule of a solid phase ELISA. The test scheme uses the attraction of purified antibody to the TSH particles of the solid phase fixation and a goat anti TSH-ab is in the ab-enzyme -horseradish peroxidase-conjugate. The assay model is left to respond at the same time with the two abs, follow-on the TSH particles being sandwiched between the solid phase and enzyme-linked ab.

Estimation of serum levels of free Thyroxine (fT4): Serum levels of fT4 were measured using fT4 enzyme immunoassay kit (Calbiotech, Inc.) following manufacturer's instruction. The CBI fT4 is a solid phase competitive ELISA. The samples Anti-T4 Biotin and fT4 enzyme conjugate are added to the wells coated with Streptavidin. fT4 in the patient's blood competes for binding sites with a T4 enzyme conjugate. The free particles that did not reacted are removed by washing buffer. Then, the substrate is added.

The depth of colour is relative to the fT4concentration in inverse way.

Estimation of serum levels of anti-TPO antibody (IgG): Serum levels of anti-TPO-ab were measured using highly sensitive anti-TPO-ab (IgG) ELISA Kit (Epitope Diagnostics, Inc., Dusseldorf, Germany) following manufacturer's instruction. The test uses the streptavidin coated microplate based enzyme immunoassay principal. The autoantibody reaction started after the addition of a biotinylated human TPO antigen. A horseradish peroxidase conjugated rabbit IgG specific ab was put in the wells. Immune-complex of solid-phase attached to biotin. TPO – human anti-TPO-ab–HRP-conjugated tracer antibody was created. HRP-conjugated tracer antibody leap to wells then incubated with a substrate solution.

Genotyping of TPO gene polymorphisms (Thr^{725}Pro of Exon 12 and Arg^{386}His of Exon 8): All the reagents were highly purified analytical PCR-materials. All the tubes, tips and pipettes used for DNA extraction were DNAse, RNAse free tubes to avoid contamination purchased from Gentra (Minnapolis. USA).

DNA extraction and storage: Genomic DNA was extracted from whole blood using the commercially available TIANamp Genomic DNA Kit (TIANGEN Biotech (Beijing) Co., Ltd), as described in the user manual. The DNA concentration was calculated by adding 20 µl of each extracted DNA sample to 1 ml of deionized water in quartz cuvet and measured the sample absorbance at 260 wavelengths using Milton Roy Spectronic 3000 Array. The DNA concentration of 50 µg/ml is indicated with the absorbance of 1.0 at 260 nm.

Polymerase chain reaction (PCR): restriction fragment length polymorphism (PCR-RFLP) was applied for detection of Thr^{725}Pro in the exon 12 according to **Hedayati et al,** [12] and Arg^{386}His of the Exon 8 of TPO gene according to **Hashemipour et al.** [19] **(Table 1)**

Table (1): Primers sequence and PCR conditions for Thr^{725}Pro of exon 12 and Arg^{386}His of exon 8 TPO gene polymorphism.

TPO gene polymorphism	Primer sequence (5'-3')	PCR protocol
Thr^{725}Pro of exon 12	F: 5′- agagtcttacaa agg gtg cac-3′ R: 5′- aag tac ctg gga gag aga agc-3′	-1 cycle at 95 ˚C for 5 min. -35 cycles at 95 ˚C for 45sec, 60 ˚C for 1min and 72 ˚C for 1 min. -1 cycle at 72 ˚C for 5 min.
Arg^{386}His of exon 8	F: 5′-ctg tct cgg gtc atc tgt g-3′ R: 5′-gta acg tgg tgt gag agg aga c-3′	-1 cycle at 95 ˚C for 10 min. -30 cycles at 95 ˚C for 50 sec, 60 ˚C for 40 sec and 72 ˚C for 30 sec. -1 cycle at 72 ˚C for 5 min.

The PCR was carried out in a final volume of 25 µl containing 100 ng of template DNA, 1.0 µM of each primer (TIANGEN BIOTECH (BEIJING) CO., LTD) and 12.5 µl of 2x Taq PCR Master Mix (TIANGEN BIOTECH (BEIJING) CO., LTD). Negative control: 20µl reaction tube containing all contents except the DNA extract was included in each run.

Restriction digestion reaction: PCR products of 205 base pair length (for Thr^{725}Pro) and 678-base pair length (for Arg^{386}His) of TPO gene polymorphisms were subjected to digestion with CutSmart™ , Recombinant, Time-Saver™ BsaJI (Bacillus stearothermophilus J695) and SacII (Streptomyces achromogenes) restriction enzymes (New England Biolab, Inc).The digestion reaction volume was 30 µl including; 10 µl of PCR product, 3 µl of 10X CutSmart® Buffer, 1 µl of FastDigest® enzyme and 16 µl of water (nuclease- free). The digested samples were left at 37°C in a heat block for 20 minutes. Construction of restriction map was done by highlighting the fragments,

counting the chosen target SNPs, and then selects the restriction enzymes from Rebase of the online program NebCutter. The sample products were detected on 4% agarose using Submarine gel electrophoresis system (Pharmacia Biotechnology by SEMKO, Sweden) and submarine chamber (Maxicell, EC360, M-E-C Co.St Florida, USA); then the gel was tested under ultraviolet trans-illumination (Heralab Gmb laborgeratetransilluminator, Germany) with 100 bp-Sizer^{TM} DNA marker (NtRON Biotechnology, Seongnam-si, Gyeonggi-do, 462-120, Korea)

Statistical analysis: The collected data were analyzed by the aid of the statistical package for social sciences version 13 (SPSS Inc., Chicago, USA).

RESULTS

Demographic data of patients and healthy controls: The controls' ages ranged from 25 - 51 years. The mean of their ages was 37.44 ± 6.59 years. The study included 5 males and 20 females. The autoimmune hypothyroid patients' ages ranged from 22 - 45 years. The mean of their ages was 34.76 ± 7.67 years. The study included 3 males and 22 females. There was no statistical significant difference as regard age and sex in the controls as compared to hypothyroid patients (P= 0.184 and 0.701 respectively). Hypothyroid patients exhibited varied clinical manifestations. Family history was recorded in 48% of cases compared with only 4% in healthy control group (P<0.0001). The mean value ± SD of BMI was 25.76±2.76 in control group and 33.12±8.65 in hypothyroid patients. The associated clinical manifestations of hypothyroidism were goiter (68%), obesity (60%), lethargy (64%), muscle cramp (56%), cold intolerance (64%) and constipation (56%). The present study revealed a significant association of hypothyroidism with family history (P< 0.001), obesity (P=0.047) lethargy (P= 0.023), muscle cramps (P=0.045) and constipation (P=0.047) .while no statistical significances were detected for other recorded clinical symptoms.

Serum levels of thyroid-stimulating hormone (TSH): The TSH levels were significantly high in hypothyroid patients (P<0.0001). In controls mean value ±SD serum TSH levels were 2.28 ±1.38 µIU/ml, as compared to 25.32 ± 6.95 µIU/ml in cases. **(Figure 1)**

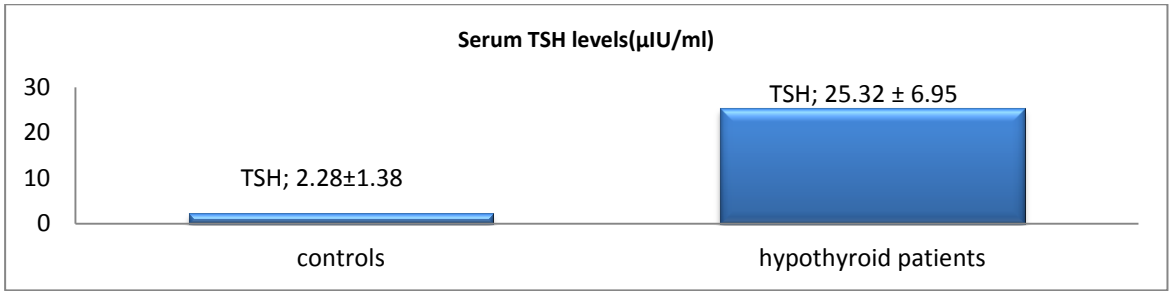

Figure (1): The mean values ±SD of TSH levels (µIU/ml) in hypothyroid patients (n=25) and in controls (n=25).

Serum levels of Free Thyroxin (fT4): In controls, mean value ±SD of serum fT4 was 1.27 ± 0.11 ng/dl as compared to 0.34 ± 0.21 ng/dl in hypothyroid patients with a highly significant difference (P <0.0001) **(Figure 2)**

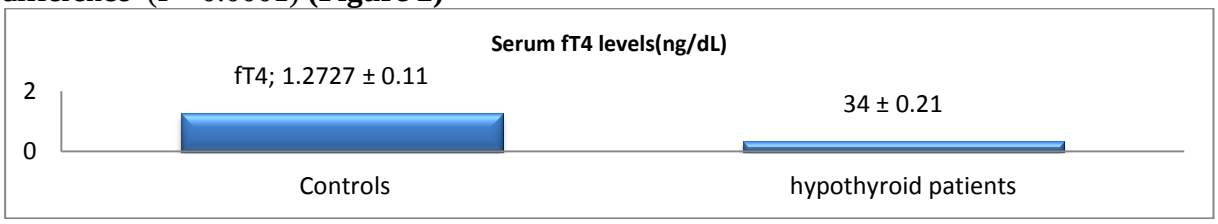

Figure (2): The mean values ±SD of fT4 levels (ng/dl) in hypothyroid patients (n=25) and in controls (n=25).

Serum levels of anti-TPO-ab: The mean value ±SD of the anti TPO-ab levels in selected hypothyroid patients was 322±305.3 IU/ml that showed highly significant difference (P <0.0001) when compared with control group in whom the mean value ±SD was 37.44 ± 5.72 IU/ml **(Figure 3)**

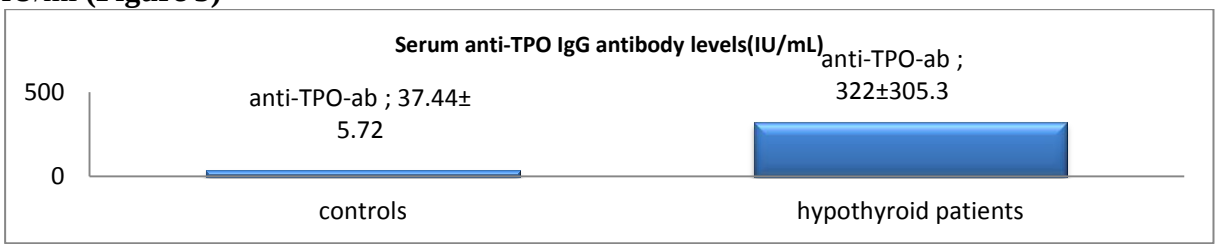

Figure (3): The mean values ±SD of the anti TPO-ab levels (IU/ml) in hypothyroid patients (n=25) and in controls (n=25).

Serum anti-TPO-ab level relationship with the different genotypes in hypothyroid patients: The mean values of serum anti TPO-ab levels in each different genotype have been calculated to find the possible association of the elevated anti-TPO-ab levels with existence of the polymorphisms.

The mean values of anti TPO-ab of each different genotype of Thr^{725}Propolymorphism in exon 12 of TPO gene are illustrated in **table (2)** and **figure (4).** The serum level of anti TPO-ab (IU/ml) of the risk genotype Pro/Pro showed a significant difference when compared with the anti TPO-ab levels (IU/ml) of Thr/ Thr genotype (P<0.0001).

Table (2): The mean values ± SD of anti TPO-ab (IU/ml) in different genotypes of Thr^{725}Pro polymorphism in exon 12 of TPO gene in hypothyroid patients (n=25).

	Thr/Thr (n=7)	**Thr/Pro (n=8)**	**Pro/Pro (n=10)**	**P value**
Serum values of anti-TPO (IU/ml)	73± 23.18	177.25± 62.81	612.1± 291.1	< 0.0001*

* Two tailed t-test

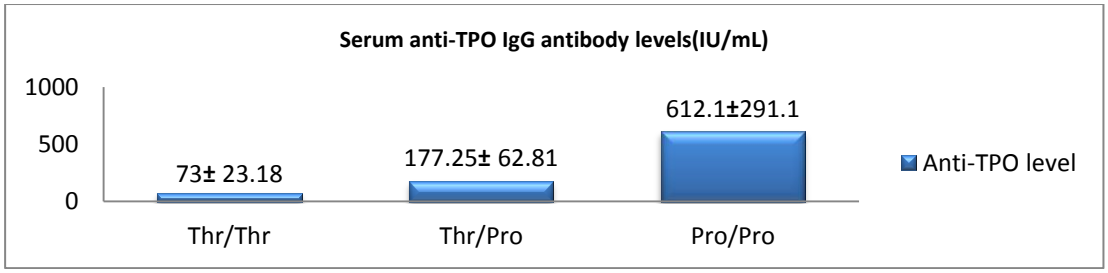

Figure (4**):** The mean values ± SD of the anti-TPO-ab levels (IU/ml) in different genotypes of Thr^{725}Pro in exon 12 of TPO gene in hypothyroid patients (n=25).

The mean values of anti-TPO-ab of each different genotype of Arg^{386}His (rs770207517) polymorphism in exon 8 of TPO gene are illustrated in **table (3) and figure (5).** The serum level of anti-TPO-ab (IU/ml) of the His/His showed no significant difference when compared with the anti-TPO-ab levels of the wild Arg/Arg genotype (P=0.529)

Table (3): The mean values ± SD of anti-TPO-ab (IU/ml) in different genotypes of Arg386His polymorphism in exon 8 of TPO gene in different genotypes in hypothyroid patients (n=25).

	His/His (n=5)	**Arg/His (n=8)**	**Arg/Arg (n=12)**	**P value**

Serum values of anti TPO (IU/ml)	286±163.42	256.33± 300.34	367.16± 355.71	0.529*

* Two tailed t-test

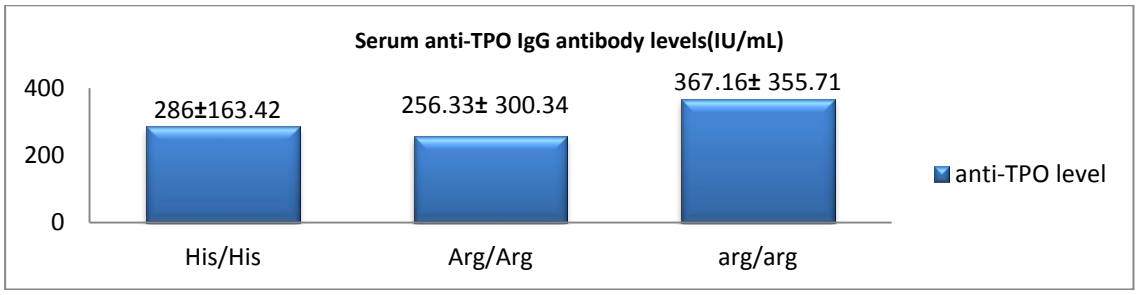

Figure (5): The mean values ± SD of the anti TPO-ab levels (IU/ml) in different genotypes of Arg^{386}His polymorphism in exon 8 of TPO gene in hypothyroid patients (n=25).

Genotyping of TPO gene polymorphisms:

1- Thr^{725}Pro polymorphism (rs732609) in exon 12 of TPO gene:

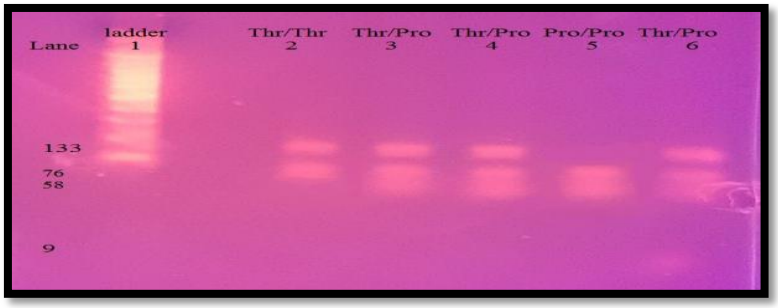

Figure (6): Agarose gel electrophoresis representing restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) analysis of Thr^{725}Pro polymorphism in exon 12 of TPO gene. Lane 1: 100 bp ladder, **Wild Thr/Thr genotype** presented in lane 2 with 133 and 76 bp. **Thr/Pro genotype** presented in lane 3,4,6: with 133, 75 and 58 bp. **Pro/Pro genotype** presented in lane 5 with 75bp and 58 bp.

The genotype frequencies were in accordance with the Hardy–Weinberg equilibrium (HWE) among both controls (P =0.92) and patients (P = 0.08).

In patients with autoimmune hypothyroidism, the frequencies of Thr /Thr, Thr/Pro, and Pro/Pro genotypes were 28, 32, and 40%, respectively; and in controls, the frequencies were 44, 44, and 12 %, respectively. The frequencies of Thr and Pro alleles in hypothyroids were 44 and 56%; and in control participates, they were 66 and 34 %, respectively. The Pro/ Pro genotype was associated with increase autoimmune hypothyroidism risk (OR= 4.889, 95% CI= 1.149-20.791, and P = 0.024). The carriage of Pro allele was significantly associated with an increased risk of autoimmune hypothyroidism (OR = 2.471, 95% CI = 1.100-5.547, and P = 0.027**) (Table 4; Figure 6,7).**

Table (4): Genotype distributions and allelic frequencies of Thr^{725}Pro polymorphism in exon 12 polymorphism in hypothyroid patients (n = 25) and controls (n = 25)

Thr^{725}Pro polymorphism	**Hypothyroid patients, n (%)**	**Controls, n (%)**	**Odds ratio (95% confidence interval)**	**P value***
Genotype				
Thr / Thr	7 (28)	11(44)	0.495(0.152-1.606)	0.238

Thr/ Pro	8 (32)	11(44)	0.599(0.189-1.898)	0.383
Pro/ Pro	10 (40)	3(12)	4.889(1.149-20.791)	0.024
Alleles				
Thr	22(44)	33(66)	----------	-----
Pro	28(56)	17(34)	2.471(1.100-5.547)	0.027

* Chi-square (χ^2) test

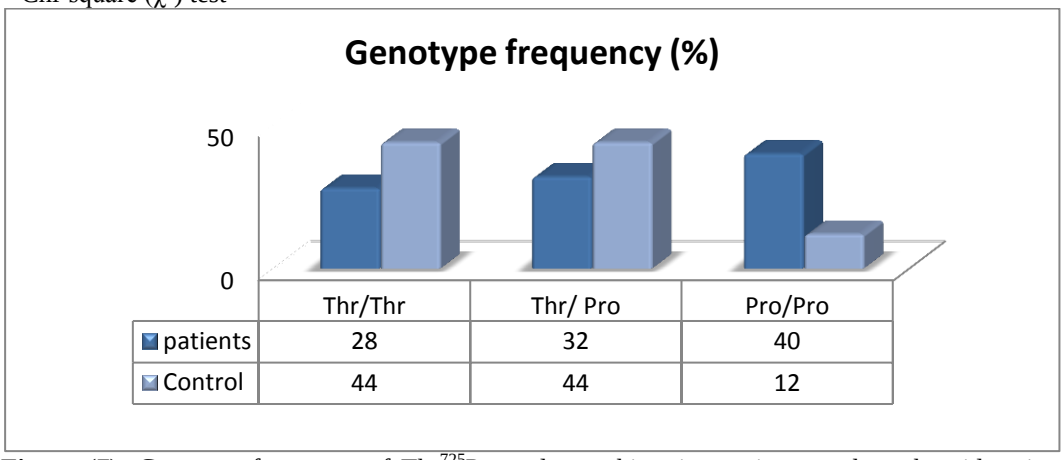

Figure (7): Genotype frequency of Thr[725]Pro polymorphism in autoimmune hypothyroid patients (n, 25) and controls (n, 25).

2-Arg[386]His (rs770207517) in the exon 8 of TPO gene:

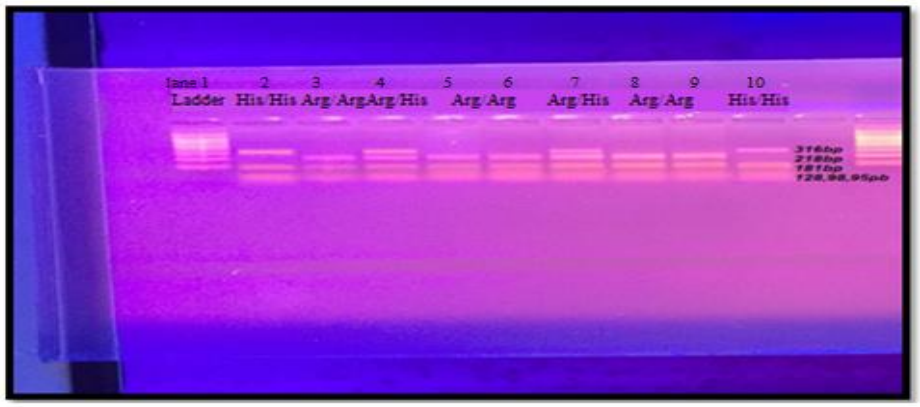

Figure (8): Agarose gel electrophoresis representing restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) analysis of Arg[386]His polymorphism in the Exon 8 of TPO gene. Lane 1: 100 bp ladder, **Wild Arg/Arg genotype** presented in lane 3, 5, 6, 8 and 9 with of 218, 181 and 128bp, **Arg/His genotype** presented in lane 4 and 7with 316, 218, 181 and 128 bp. **His/His genotype** presented in lane 2 and 10 with 316, 181 and 128 bp.

The genotype frequencies were in accordance with the Hardy–Weinberg equilibrium (HWE) among both controls (P =0.096) and patients (P= 0.127).

In patients with autoimmune hypothyroidism, the frequencies of Arg/Arg, Arg/His, and His/His genotypes were 48, 32, and 20%, respectively; and in control participates, the frequencies were 56, 28, and 16 %, respectively. The frequencies of Arg and His alleles in hypothyroids were 64 and 36%; and in controls were 70 and 30 %, respectively.

The His/His genotype showed no significant association with the occurrence of autoimmune hypothyroidism (OR = 1.312, 95% CI = 0.308-5.598, and P = 0.99). The carriage of His allele was not associated with an increased risk of autoimmune hypothyroidism (OR = 1.312, 95% CI = 0.569-3.029, and P = 0.522) **(Table 5; figure 8,9).**

Table (5): Genotype distributions and allelic frequencies of Arg^{386}His polymorphism in hypothyroid patients (n = 25) and controls (n = 25)

Arg^{386}His polymorphism	Hypothyroid patients, n (%)	Controls, n (%)	Odds ratio (95% confidence interval)	P value*
Genotype				
Arg / Arg	12(48)	14(56)	0.725(0.238-2.208)	0.572
Arg / His	8(32)	7(28)	1.210(0.360-4.065)	0.752
His / His	5(20)	4(16)	1.312(0.308-5.598)	0.99**
Alleles				
Arg	32(64)	35(70)	----------	—
His	18(36)	15(30)	1.312 (0.569-3.029)	0.522

* Chi-square test **Fisher exact test

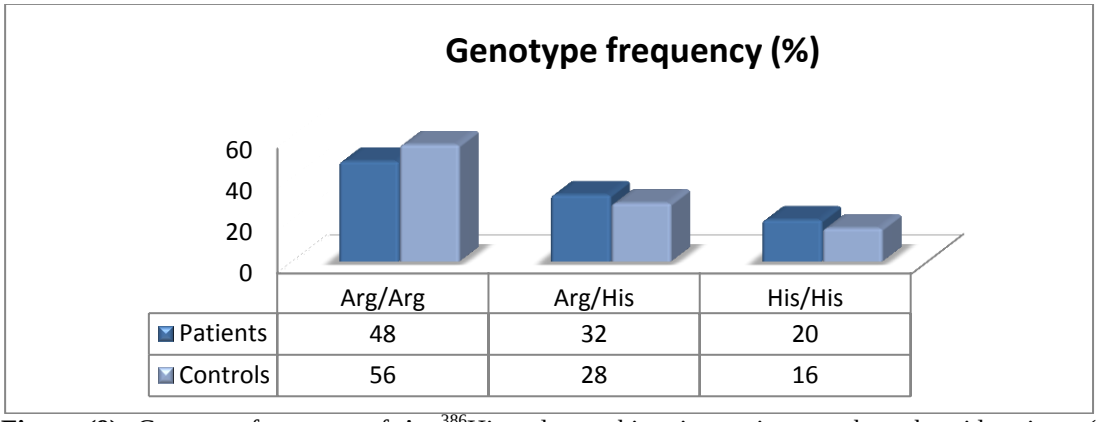

Figure (9): Genotype frequency of Arg^{386}His polymorphism in autoimmune hypothyroid patients (n, 25) and controls (n, 25).

DISCUSSION

Autoimmune hypothyroidism become the main cause of thyroid gland insufficiency in most countries all over the world.[20] Its worldwide prevalence is about 2 to 4% of women and up to 1% of men according to **Mousa et al.**.[21]

The aim of current study was to evaluate the role of TPO gene polymorphisms at two points (Thr^{725}Pro of exon 12, and Arg^{386}His of exon 8 of TPO gene) as important causes of hypothyroidism and to detect the association between anti-TPO-ab levels with different genotypes in adult Egyptian patients with autoimmune hypothyroidism, attended Endocrinology outpatient clinic of Zagazig university hospital.

An inherent limitation to all case-control studies is that matched groups may differ in respects other than just the trait of interest. We controlled for BMI, age, sex, and none of the participants had past history of diseases that may affect thyroid gland. Therefore, we believe we have at least minimized the chance that the abnormalities in thyroid function were due to anything else but the genetic polymorphism.

The demographic data of the participants in the present study revealed that the frequency of the hypothyroidism was higher in females than in males, 22 female/3male, with a male/female ratio of 1:7.3. This result is near the worldwide male: female ratio which is 1:6. [22] The mean age of patient group was 34.76 ± 7.67 years indicating that the hypothyroidism is higher in women than in men and mostly affects the middle ages.

These annotations are in agreement with the previous studies.[23],[24],[20]

Clinical assessment of hypothyroid patients was based on proper history taking, and clinical examination.[18] The laboratory diagnose of autoimmune hypothyroidism was confirmed by elevated TSH, low fT4 and positive anti TPO-ab serum level.[25] About 48% of hypothyroid patients showed positive familial history. **Balmiki et al.** [20] and **Guria et al.** [26] have reported 46.5% positivity of family history among hypothyroid patient, results that go hand in hand with our data. The family history relationship with autoimmune hypothyroidism was announced by many previous studies as **Colin et al.** [27] and **Gopalakrishnan et al..**[28]

Autoimmune hypothyroidism is a common cause of goiter.[29] Goiter in this study was detected in 68% of patients which is near to the incidence found by **Guria et al.**[26]**,** as goiter was found in 65.5%. However, **Balmiki et al.**[20] has found goitre in only 21% of their patients. This difference can be explained on the bases of difference in genetic predisposition and other factors that affect the occurrence of autoimmune diseases.[30],[31]

The present study revealed a significant association of hypothyroidism with obesity (P=0.047), lethargy (P= 0.023), muscle cramps (P=0.045) and constipation (P=0.047) which are in accordance with the known clinical presentation of hypothyroidism[18] ,while no statistical significance was detected for cold intolerance. Although cold intolerance has been strongly associated with hypothyroidism, this could be explained by the small sample size of the present study.

The elevated anti-TPO-ab level in patient group was used as an inclusion criteria in this study and there was a highly significant difference (P <0.0001) of anti TPO-ab levels in hypothyroid patients when compared with control group. Anti-TPO-ab was taken as inclusion criteria for patient group as it is the most clinically important auto antibodies produced to target the thyroid.[32] They are present in approximately 90% of autoimmune hypothyroidism.[17] The serum levels of these auto-antibodies increase in the active phase of autoimmune hypothyroidism so, they are used to follow up the treatment strategy.[17],[14]

To our knowledge, the present study is the earliest study tried to investigate the genetic basis of autoimmune hypothyroidism in adult Egyptian patients. Detection of TPO gene mutations in patients with thyroid dyshormonogenesis has been investigated by numerous groups in different countries, in Portugal [33], Netherlands [6], Argentina [34], Japan Brazil [35],[36] and China [37]. The majority of these studies aimed to detect the relation between TPO gene abnormalities and congenital hypothyroidism.

Many previous studies indentified more than 50 TPO gene mutations caused by DNA nucleotide bases deletion, insertion, or changing sequences.[38] Mutations that lead to change the normal location of the enzyme or change its three dimensional shape can affect the enzyme activity**.** Consequently, the TH production affected, leading to appearance of thyroid disorders symptoms.[15]

The present study focused on mutations of TPO gene that could change certain amino acids in the protein structure of TPO enzyme as **El Shabrawy et al.**[39] They have reported that changing certain amino acids in TPO enzyme may affect both the structure and functional activity of TPO as it can influence the all folding of the protein, affect its activity and/or its rate of degradation.

In the present study, the genotypes of the studied polymorphism showed no deviation from HWE in both patients and controls (P>0.05) indicating absence of selection bias, good sample presumption as deviations from HWE in controls can be very informative. The deviation indicates possible breach of the sample presumption

roles, mostly occurs in genotyping error in sample selection. Other causes of these deviations may occur due to coincidence, presence of protective allele, population admixture/ stratification or endogamy. [40]

The first point of polymorphism included in this study was the polymorphism at $Thr^{725}Pro$ in exon 12 of TPO gene which leads to substitution of threonine to proline amino acid. Genotyping revealed that the carriage of Pro/Pro genotype is associated with 4.889 fold increase of having hypothyroidism. Pro allele found to be significantly associated with an increased risk of autoimmune hypothyroidism (OR = 2.471, 95% CI = 1.100-5.547, and P = 0.027).

Alterations of Threonine residue to Proline residue in $Thr^{725}Pro$ may possibly disrupt the secondary structure of TPO protein. This can be supported on the base of chemical difference between the two amino-acids. Threonine amino acid is hydrophilic, polar, uncharged amino acid. It acts as a phosphorylation site of protein which is important for protein activation process.[41] While proline is an imino acid. It has a distinctive cyclic structure. The proline amino acid backbone is locked with dihedral angle at approximately -75°. This is the cause of the exceptional conformational rigidity of proline when compared to other amino acids. As a result, proline greatly affects protein architecture upon folding.[42] The presence of proline instead of threonine within the protein skeleton disrupts the usual protein secondary structural structure, such as alpha helixes and beta sheet. [20] Moreover, there are no hydrogens on the amide group proline so; it cannot act as a hydrogen bond donor when compared with threonine. [43]

In agreement with our study, two studies in Indian population reported similar results, **Guria et al.**[26] found that Pro/Pro genotype can be considered as the risk genotype (OR=1.45, CI=1.09-1.92, P= 0.01) while the Pro allele can be the risk allele (OR=2.15, CI=1.24-3.71, P=0.006). Also, **Balmiki et al.**[20] considered Pro/Pro as the risk genotype in Indian population (P=0.01) and Pro allele is a risk allele (OR= 1.45, CI =1.09-1.92, P=0.006).In study on Iranian population, Pro/Pro genotype was observed in 71.2% in their patients with high significance (P <0.001).[12]

We also tried to find the relation between $Thr^{725}Pro$ polymorphism and anti-TPO-ab level in our hypothyroid patients. The serum level of anti-TPO-ab of the risk genotype Pro/Pro showed a significant difference (P< 0.0001) when compared with the anti-TPO-ab levels of the wild Thr/ Thr genotype. Similar results were concluded in previous studies on congenital hypothyroidism [45] and in adult autoimmune hypothyroidism. [20],[44],[12]

The change in protein structure of the TPO can stimulate the immune system.[44],[39] So, we hypothized that the genetic variations in TPO protein sequence can change its structure, consequently TPO enzyme acts as a foreign body. This will stimulate the immune system to produce antibodies against it.

The anti-TPO-ab can damage thyroid cell by both antibody dependent cell cytotoxicity and complement activation**.** [17] In chronic autoimmune thyroiditis interferon-γ produced by activated CD^{4+} T-cells cause activation of MHC class II molecules. Thus, the presence of anti-TPO-ab can expand the auto-reactive T-cell repertoire and prolongs the inflammatory response. [46]

The second point of polymorphism included in this study was the polymorphism at $Arg^{386}His$ in exon 8 of TPO gene which leads to change of histidine to arginine amino acids. This polymorphism showed insignificant association with hypothyroid patients. The His/His genotype of $Arg^{386}His$ (OR = 1.312, 95% CI = 0.308-5.598, and P = 0.99) as well as His allele (OR = 1.312, 95% CI = 0.569-3.029, and P = 0.522). In accordance with this result, **Guria et al.**[26]

and **Hedayati et al.**[12] found that this point was not polymorphic and there were no association of changing the amino acid in that point of exon 8.

Contradictory to our results, **Hashemipour et al.**[19] in Iranian population and **Bikker et al.**[47] in Netherlands of Holland have found inactivation mutations in exon 8 were associated with hypothyroidism. They claimed that such mutations can be the result in thyroid dyshormonogenesis in their patients. The discrepancies between the studies can be due to ethnic difference.

Investigating the relation of $Arg^{386}His$ with anti-TPO-ab, the serum level of anti-TPO-ab (IU/ml) of the His/His showed no significant difference (P=0.529) when compared with the anti-TPO-ab levels of the wild Arg/Arg genotype.

This can be explained on the molecular level by the fact that both arginine and histidine are basic, hydrophilic, polar amino acids. Guanido group of arginine and imidazole group of histidine both can act as a buffer (proton acceptor or donor) for chemical reactions. Because of chemical similarity between both amino acids, we expect that the change in amino acid, arginine to histidine in the TPO gene will not affect the structure of the enzyme and do not affect its activity.[42] These facts explain that $Arg^{386}His$ of exon 8 polymorphism was not associated with the anti TPO-ab level. **Hedayati et al.**[12] agreed with this result.

Although this study revealed some statistically significant results, we admit that our conclusions are still incompletely developed since first; the sample size was rather small and did not have sufficient statistical power to finding out the actual association. Second, the hypothyroid patients were elected only from the Zagazig town, and other areas in Egypt where not included. Third, ethnic differences may be an imperative aspect that may influence genetic studies of the selected disease.

As two gene polymorphisms detection are insufficient to present the complete clarification of the genetic constituent for autoimmune hypothyroidism, future studies are essential to clarify the possible association of other TPO polymorphisms with hypothyroidism. This understanding may be reflected clinically by prediction of patients with hypothyroidism apply genetic which may allow early detection and better control of such patients.

Conclusion: In sum, it is the first study, in our knowledge, that found association of $Thr^{725}Pro$ of exon 12 of TPO gene with autoimmune hypothyroidism and correlated the anti-TPO-ab levels with different genotypes in hypothyroid patients. Also, there was no association of $Arg^{386}His$ of exon 8 of TPO gene with autoimmune hypothyroidism, and no relation was found between the anti TPO-ab levels in hypothyroid patients and its different genotypes in Egyptian population. However, this field needs largerer case-control studies with more sample size. There is a need for studies of other nationalities and ethnicities to verify the current conclusions.

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