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Research Article

TPO Gene Expression in Relation with Promoter SNPs in Iraqi Patients with Hyperthyroidism

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Abstract

Background: Thyroid peroxidase (*TPO*) gene mutations are one of the most common causes of thyroid disorders. ***Objective***: To investigate the effect of genetic polymorphisms in the *TPO* promoter region on gene expression in early-diagnosed hyperthyroid patients. ***Methods***: Genomic DNA was extracted from 100 blood samples (75 hyperthyroid patients and 25 healthy controls), then the *TPO* promoter region was amplified and sequenced for genotyping rs2071399, rs2071400, and rs2071403 SNPs. Total RNA was also isolated, and cDNA synthesis was performed to determine quantitatively the expression of *TPO* by using qPCR. The level of TPO antibodies in serum was determined by using an enzyme-linked immunosorbent assay (ELISA). ***Results***: The prevalence of hyperthyroidism in women was significantly higher than in men, as were serum levels of TPO-Abs. There was a significant increase in serum TPO-Abs in hyperthyroid patients (235.29 IU/ml) compared with healthy controls. Genotypes of three SNPs (rs2071399 G/A, rs2071400 C/T, and rs2071403 A/G) in the *TPO* promoter region were *TPO* rs2071399 AG and GG, and rs2071400 CT and TT genotypes were more frequent in hyperthyroid patients. There are no significant differences between rs2071403 polymorphic and non-polymorphic genotypes among hyperthyroid patients and healthy controls. The rs2071399 G/A and rs2071400 C/T gene promoter polymorphism significantly down-regulated constitutive *TPO* gene expression in hyperthyroid patients, but rs2071403 A/G has no major effect on gene expression. ***Conclusion***: There was an association between the mutation in the promoter region of TPO and the incidence of hyperthyroidism.

Keywords: Genetic polymorphisms, Hyperthyroidism, Promoter SNPs, *TPO* gene expression.

التعبير الجيني لجين الثايرويد بيروكسيديز وعلاقته بالتغايرات الوراثية في منطقة الحفاز لدى مرضى فرط نشاط الغدة الدرقية العراقيين

الخلاصة

الخلفية: تعد طفرات جين بيروكسيديز الغدة الدرقية (TPO) أحد الأسباب الأكثر شيوعا لاضطرابات الغدة الدرقية. **الهدف**: التحقيق في تأثير تعدد الأشكال الجينية في منطقة حفاز جين TPO على التعبير الجيني لدى مرضى فرط نشاط الغدة الدرقية الذين تم تشخيصهم حديثا. **الطريقة**: تم استخراج الحمض النووي الجينومي من 100 عينة دم (75 مريضا بفرط نشاط الغدة الدرقية و 25 من الأصحاء)، ثم تم تضخيم منطقة حفاز TPO وتسلسلها للتنميط الجيني rs2071399 و rs2071400 و rs2071403 SNPs. كما تم عزل إجمالي الحمض النووي الريبي، وتم إجراء تخليق cDNA لتحديد تعبير TPO كميا باستخدام qPCR وتم تحديد مستوى الأجسام المضادة في المصل باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم. **النتائج**: كان انتشار فرط نشاط الغدة الدرقية لدى النساء أعلى بكثير من الرجال وكانت هناك زيادة كبيرة في مصل TPO-Abs في مرضى فرط نشاط الغدة الدرقية مقارنة بمجموعة السيطرة الأصحاء. كانت الأنماط الجينية لثلاثة SNPs (rs2071399 G/A و rs2071400 C/T و rs2071403 A/G) في منطقة حفاز TPO هي TPO rs2071399 AG وGG ، وكانت الأنماط الجينية rs2071400 CT و TT أكثر تواترا في مرضى فرط نشاط الغدة الدرقية. لا توجد فروق ذات دلالة إحصائية بين الأنماط الجينية متعددة الأشكال وغير متعددة الأشكال rs2071403 بين المرضى والأصحاء. أدى تعدد الاشكال الوراثية لمنطقة حفاز للجين rs2071399 G/A وrs2071400 C/T إلى انخفاض كبير في التعبير الجيني TPO في مرضى فرط نشاط الغدة الدرقية، ولكن rs2071403 A/G ليس له تأثير كبير على التعبير الجيني. **الأستنتاج**: كان هناك ارتباط بين الطفرة في منطقة الحفاز ل TPO وحدوث فرط نشاط الغدة الدرقية.

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INTRODUCTION

Thyroid peroxidase (TPO) is an enzyme found in the apical membrane of thyrocytes, specialized cells in the thyroid gland. It plays a critical role in the synthesis of thyroid hormones. TPO catalyzes the oxidation of iodide and its incorporation into tyrosine residues on thyroglobulin, leading to the formation of active thyroid hormones [1,2]. The TPO gene is located on chromosome 2p25 and encodes a protein composed of 933 amino acids. It is organized into 17 exons containing a coding sequence of 3 kilobases (kb) [3]. Mutations in the TPO gene can lead to defects in iodide organification, which can result in impaired thyroid hormone production [2,4]. These defects can be total (TIOD) or partial (PIOD), meaning they can completely or partially inhibit the iodination process. This disruption in thyroid hormone production can lead to various thyroid disorders. The TPO gene is known to have a high degree of genetic diversity, with more than 170 mutations recorded in the Human Gene Mutation Database (HGMD) as of 2021. These mutations can have varying effects on TPO function and thyroid hormone synthesis [4–5]. Understanding the genetic basis of thyroid disorders related to TPO mutations is crucial for diagnosis and treatment. It highlights the complexity of thyroid hormone regulation and the importance of TPO in maintaining thyroid function. Additionally, it underscores the value of genetic testing in identifying and managing thyroid-related conditions, particularly in cases of congenital hypothyroidism associated with TPO genetic defects. The regulation of the TPO gene primarily occurs at the transcriptional level, with thyroid-stimulating hormone (TSH) from the pituitary gland serving as the primary hormonal regulator of TPO gene expression [6]. Interestingly, the administration of iodide, whether in physiological or high doses, leads to a reduction in TPO mRNA levels [7]. This reduction is attributed to the production of a compound known as 2-iodohexadecanal (2-IHDA), which exerts its influence by modulating the association of thyroid transcription factors with the TPO gene. Furthermore, iodide not only inhibits the response of thyrocytes to TSH but also results in the down-regulation of genes that are typically positively regulated by this hormone. Moreover, interferon IFN α and β, along with IL-1a and IFN-g, have the capacity to downregulate both TSH-stimulated TPO expression and the release of T4 from primary human thyrocytes [8]. Studying the TPO gene mutation of patients diagnosed with autoimmune thyroid diseases (Hashimoto's thyroiditis and Graves' disease) has been interesting in many different countries around the world, like Argentina, Portugal, the Netherlands, Japan, China, Iran, Iraq, and many other countries [9]. Studies try to find specific mutations that may occur in one or several positions of the TPO gene and their effect on gene function. This study aimed to determine the relationship between naturally occurring variants of TPO promoter regions and gene expression in newly diagnosed hyperthyroid patients and provide valuable information for clarifying the molecular mechanisms underlying the pathogenesis caused by TPO mutations. Understanding how these factors influence TPO expression and thyroid hormone release can shed light on the complex interplay involved in thyroid disorders associated with TPO mutations.

METHODS

Study design and samples

A cohort of 75 newly diagnosed hyperthyroid patients was enrolled at the National Diabetes Center, Mustansiriyah University, between July and November 2021. The inclusion criteria comprised individuals with elevated mean levels of T3 (2.07 nmol/L) and T4 (14.02 µg/dl), along with decreased mean levels of TSH (0.17 µIU/ml) at the time of diagnosis. Patients with hyperthyroidism who also had concurrent diabetes mellitus, hypertension, cardiovascular diseases, or were pregnant were excluded from the study.

DNA Extraction and Sequencing

Genomic DNA was extracted from the peripheral blood samples collected from recruited patients by using the Zymo Research Quick-DNA™ Miniprep Kit. Quality and quantity of extracted DNA was assessed by electrophoresis and spectrophotometry using a nanodrop spectrophotometer. The promoter regions covering rs2071399, rs2071400, and rs2071403 SNPs were targeted by amplification.

PCR Primers

TPO promoter regions were amplified using specific primers indicated in Table 1. These primers were purchased in lyophilized samples of different picomol concentrations. PCR was performed in a thermocycler (Applied Biosystems). The reaction mixture (25 µL) consisted of 40–100 ng of genomic DNA, 1.5 mM MgCl2, 100 µM of each dNTP, 0.4 µM of each primer, and 0.5 U of Taq DNA polymerase. The amplification protocol involved denaturation at 95°C for 30 seconds, annealing at a temperature between 60 and 62 °C for 30 seconds, and extension at 72°C for 30 seconds, for a total of 25 cycles. Subsequently, the PCR products were subjected to direct sequencing using the Sanger sequencing method, conducted by Macrogen Company in Korea. Amplicons of varying lengths, ranging from 212 bp to 448 bp, were obtained during each sequencing run. The obtained sequences were aligned

with corresponding sequences of the TPO gene available on the National Center of Biotechnology Information (NCBI) using Bioedit software.

Table 1: Oligonucleotide primers used for amplification *TPO* promoter regions

SNP	Sequence (5'→3')	Tm (°C)	Product size (bp)	Reference
RS2071399	F: CCCTGCTCTTCAGAGCTTGT R: GGTCACCATGACTGCATTCC	62	417	
RS2071400	F: GCATGAAGAGGCTCCAAGTC R: GCATTCTCCCCTCAATCCTC	62	212	Tomari *et al.* [11]
RS2071403	F: ACATTCTGTCCCCACGAAGA R: AAGCAGGATAGCACCAGTGA	60	448	

Gene expression analysis

Total RNA was extracted by using TRIzol reagent (Invitrogen) from blood samples of hyperthyroid patients and healthy controls. The cDNA was made with reverse transcriptase and was diluted 30 times with deionized water before it was used as a template in the qRT-PCR SYBER green assay. The quantitative reaction was performed on a real-time PCR system using the Go*Taq*® qPCR Master Mix kit (Promega) with forward (5'-CCGAGCAGCAGAGATAATGG-3') and reverse (5'-CCGTTGGATGCTGTGATTGT-3') primers. To synthesize complementary DNA (cDNA), 1 mg of total RNA was employed, following the RIzol™ Reagent Protocol. The mixture underwent incubation at 25 °C for 10 minutes and at 42 °C for 60 minutes, followed by heating to 99 °C for 5 minutes, and then storage at -20 °C. PCR amplification utilized 5 mL of cDNA, and the samples were subjected to 41 cycles of amplification with the following PCR conditions for the TPO genes: denaturation at 94 °C for 1 minute, annealing at 62 °C for 1 minute, and extension at 72 °C for 1 minute for 40 cycles. The final cycle involved an extension step at 72 °C for 7 minutes. Expression of the *GAPDH* housekeeping gene was estimated for normalization of the mRNA level of the *TPO* gene by using Qiagene Rotor gene qRT-PCR, Germany. *TPO* fold change expression was determined by the employment of TransStart Top Green Super Mix by Transgene Biotech, China.

Statistical analyses

The Statistical Analysis System (SAS) program, as of 2018, was employed to assess the impact of various factors on the study parameters. To compare means significantly, a *t*-test was employed. For comparing percentages significantly, the Chi-square test was applied, with significance levels set at $p<0.05$. In this study, odds ratios and confidence intervals (CIs) were also calculated. Parametric variables were presented as mean±SEM, while group variables were presented as percentages.

RESULTS

Seventy-five subjects newly diagnosed as hyperthyroid patients were enrolled in this study, in addition to 25 healthy controls of both genders. The hyperthyroidism group includes 64 females (85.33%) and 11 males (14.67%), while the control group includes 20 females (80%) and five males (20%), as indicated in Table 2.

Table 2: Distribution of the studied group according to gender

Group	No.	Male *n*(%)	Female *n*(%)	*p*-value
Patients	75	11(14.6)	64(85.3)	0.0001
Control	25	5(20.0)	20(80.0)	0.0027
p-value	--	0.133	0.0001	---

According to these results, the prevalence of hyperthyroidism in women was significantly higher ($p< 0.001$) than in male patients. In this study, serum levels of TPO-Abs were investigated in hyperthyroid patients in comparison with healthy controls. The results in Table 3 clearly demonstrate a significant elevation ($p<0.01$) in TPO-Abs within the serum samples of hyperthyroid patients, measuring 235.29 IU/ml. In contrast, the TPO-Abs levels in healthy controls were notably lower at 20.7 IU/mL. It is noteworthy that both values remain within the normal range (less than 35 IU/ml), despite the significant difference observed. According to these results, TPO-Abs is highly predictive of thyroid autoimmunity and hyperthyroidism.

Table 3: Serum levels of TPO-Abs and TR-Abs in hyperthyroid patients and healthy control

Group	Mean±SEM
	TPO-Abs (IU/ml)
Patients	235.29±17.37
Control	20.76±0.87
t-test	54.177
p-value	0.0001

The results in Table 4 reveal a significant increase ($p<0.001$) in the frequency of G carriers (AG and GG genotypes) among hyperthyroid patients compared to the control subjects. Additionally, the G allele frequency was notably higher ($p=0.005$) in hyperthyroid patients when compared to control subjects, using the C allele as the reference allele.

These findings suggest a clear association between this polymorphism and the incidence of hyperthyroidism.

Table 4: Genotypes and allele frequency of *TPO* rs2071399 polymorphism in hyperthyroid patients and healthy controls

Genotype	Patients *n*(%)	Control *n*(%)	χ^2	Odd ratio (OR)	Confidence interval (CI 95%)
AA	3(12)	13(52)	6.2	1	Reference
AG	14(56)	10(49)	0.66	1.9	(0.6-5.8)
GG	8(32)	2(8)	3.6	5.87	(1.10-31.23)
Allele frequency					
A	20(40)	36(72)		$\chi^2 = 10.5$	
G	30(60)	14(28)		*p*-value = 0.005	

As delineated in Table 5, the results demonstrate a noteworthy increase ($p<0.001$) in the frequency of T carriers (CT and TT genotypes) among hyperthyroid patients in comparison to control subjects. Furthermore, the T allele frequency exhibited a significant elevation ($p=0.0009$) among hyperthyroid patients when contrasted with control subjects, with the C allele serving as the reference allele. These findings underscore a clear association between this polymorphism and the occurrence of hyperthyroidism.

Table 5: Genotypes and allele frequency of *TPO* rs2071400 polymorphism in hyperthyroid patients and healthy control subjects

Genotype	Patients *n*(%)	Control *n*(%)	χ^2	Odd ratio (OR)	Confidence interval (CI 95%)
CC	4(16)	17(68)	8.04	1.00	Reference
CT	14(56)	6(24)	3.2	4.03	(1.19-13.4)
TT	7(28)	2(8)	2.76	4.03	(1.19-13.4)
Allele frequency					
C	22(44)	40(80)		$\chi^2 = 14.025$	
T	28(56)	10(20)		*p*-value = 0.0009	

Table 6 showed that the GG genotype frequency in hyperthyroid patients was not significantly different than in control subjects. The dominant G allele frequency was not significantly different in hyperthyroid patients than in control subjects, while the recessive G allele was significantly higher ($p<0.001$) in hyperthyroid patients than in control subjects. This is the first study in Iraq about the relationship between *TPO* gene expression and *TPO* promoter regions in hyperthyroid patients. RT-PCR was used to determine the level of *TPO* mRNA transcriptional levels in case and control subjects.

Table 6: Genotypes and allele frequency of TPO rs2071403 polymorphism in hyperthyroid patients and healthy controls subjects

Genotype	Patients *n*(%)	Control *n*(%)	χ^2	Odd ratio (OR)	Confidence interval (CI 95%)
AA	2(8)	8(32)	3.6	1.00	Reference
AG	16(64)	9(36)	1.96	3.02	(0.94-9.53)
GG	7(28)	8(32)	0.06	0.825	(0.24-2.78)
Allele frequency					
A	20(40)	25(50)		$\chi^2 = 5.262$	
G	30(60)	25(50)		*p*-value = 0.06	

Figure 1 showed that there is a significant decrease ($p<0.01$) in *TPO* gene expression in hyperthyroid patients compared with healthy controls.

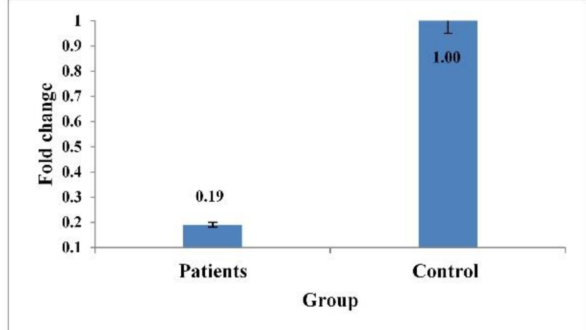

Figure 1: Fold change expression of *TPO* in hyperthyroid patients and healthy controls.

This may be due to the genetic polymorphisms, especially rs2071399 and rs2071400, whose polymorphic genotypes are significantly higher ($p<0.001$) in hyperthyroid patients than in control subjects.

DISCUSSION

Gender-related aspects within hyperthyroid patients were investigated. The findings of the present study indicate a significant disparity ($p<0.01$) between male and female hyperthyroidism patients. Specifically, females exhibited a significantly higher prevalence than males. It is noteworthy that males and females exhibit varying predispositions to thyroid dysfunction, with various forms of thyroid dysfunction being more prevalent in women. Furthermore, the overall incidence

of thyroid dysfunction tends to increase with age in both genders, particularly in women [10]. The results of the current study showed that 80% of hyperthyroid patients are female, which agrees with Mazeh *et al.* (2012) [11], who found that the hyperthyroidism prevalence ratio was 3:1 female to male, depending on population and detection methods. While Castello and Caputo (2022) [12] found that the incidence ratio was seven times higher in females than males, gender plays a key role in the incidence rate. On the other hand, there are many studies done in Iraq concerned with thyroid dysfunction development and its correlation with gender, such as those found by Al-Mofarji (2014) [13], Amin *et al.* (2018) [14], and Khalaf (2020) [15]. Each of these studies found that there is an increase in the prevalence of thyroid dysfunction in females compared to males in the Iraqi population. The current study showed that there is an association between serum levels of TPO-Abs and homopolymorphic alleles for all the detected SNPs except rs1126797, which means that these SNPs stimulate the synthesis of TPO-Abs in hyperthyroid patients due to the significant increase in serum levels of TPO-Abs in patients carrying these genotypes. These findings are similar to those obtained by Khoshi *et al*. (2017) [16], who found that serum levels of TPO-Abs increased significantly in hypothyroid patients who have a polymorphic allele. Genetic analysis of the *TPO* promoter region in both cases and controls was carried out to investigate the gene polymorphisms and the incidence of hyperthyroidism. In this study, we screened and identified *TPO* gene mutations in *TPO* promoter regions in Iraqi patients with hyperthyroidism. The genetic polymorphism is due to variation in *TPO* promoter regions relative to the transcription start site, affecting gene expression. Results of the current study showed that there is a significant relationship ($p<0.01$) between rs2071399 polymorphism and the susceptibility to hyperthyroidism in a dominant polymorphic G allele and the recessive model of inheritance allele (G) in hyperthyroid patients and healthy subjects. According to these results, the polymorphic G allele is a risk factor for the incidence of hyperthyroidism, and carriers of this allele have a higher risk of presenting hyperthyroidism (relative to carriers of the A allele). On the other hand, results also showed that there is a significant relationship ($p<0.001$) between rs2071400 polymorphism and the incidence of hyperthyroidism in the dominant polymorphic T allele and the recessive model of inheritance allele (T) in both groups. According to these results, the polymorphic T allele is also a risk factor for the incidence of hyperthyroidism. These findings are similar to those obtained by Tomari *et al.* [17] and Hernado [18]. These results are also similar to those obtained by Ahmed *et al.* [19], who found that the TT polymorphic genotype of rs2071400 C/T and T allele were significantly more frequent in subclinical hypothyroidism patients over hypothyroidism than in the control group. While the current results showed that there is no significant relation between the rs2071403 SNP and the incidence of hyperthyroidism in the studied group due to the non-significant difference in the dominant polymorphic G allele among hyperthyroid patients and healthy controls, This result mentioned that the dominant polymorphic allele G is not a risk factor for the incidence of hyperthyroidism, while the recessive model of the inherited G allele is a risk factor for the susceptibility to hyperthyroidism. These results are similar to those obtained by Renaguli *et al.* [20], who found that rs2071403 *TPO* polymorphism was associated with hypothyroidism under the additive model (AA/GG) and dominant model. The current study showed that there is an association between serum levels of TPO-Abs and homopolymorphic alleles for all the detected SNPs except rs1126797, which means that these SNPs stimulate the synthesis of TPO-Abs in hyperthyroid patients due to the significant increase in serum levels of TPO-Abs in patients carrying these genotypes. These findings are similar to those obtained by Khoshi *et al*. (2017) [16], who found that serum levels of TPO-Abs increased significantly in hypothyroid patients who have a polymorphic allele. Gene expression is a fundamental life process that provides a bridge between information encoded within a gene and a final functional gene product, such as a protein or non-coding RNA [21]. The naturally occurring sequence variation in *TPO* promoter regions may result in differential expression of *TPO* in hyperthyroid patients. The results of the current study showed that *TPO* gene expression decreased five times in hyperthyroid patients. This downregulation may be due to genetic polymorphisms in the promoter region (as a regulatory region) of the gene, especially rs2071399 and rs2071400, which were significantly associated with the incidence of hyperthyroidism. These two functional SNPs are associated with altered gene expression according to the low level of mRNA transcript of the gene detected by RT-PCR as autosomal and appear to conform to a pattern of autosomal dominance of the rs2071399 G allele and the rs2071400 T allele. There are many studies on genetic polymorphisms of *TPO* and their effect on gene expression or on the activity of the TPO enzyme, but the vast majority of these studies investigate this association in the hypothyroidism group but not in hyperthyroidism [21]. Furthermore, SNPs located in the promoter region have a strong effect on the level of *TPO* gene expression and cause a strong association between rs2071403 *TPO* gene polymorphism and downregulation of *TPO* gene expression [22].

Conclusions

The high prevalence of mutations in *TPO* promoter regions (rs2071399, rs2071400, and rs2071403) leads to downregulation of gene expression and plays a pivotal role in the development of hyperthyroidism.

Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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