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The Impact of rs945006 Polymorphism on Levothyroxine Therapy

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Abstract

Levothyroxine (L-T4) replacement therapy is affected by a variety of factors. Long after the treatment, many patients suffer from persistent symptoms of hypothyroidism. Thyroxine (T4) is transformed by the deiodinase type 3 enzyme into reverse triiodothyronine (rT3) and triiodothyronine (T3) into diiodothyronine (T2). As a result, this enzyme is crucial for maintaining balanced levels of thyroid hormone homeostasis in the body. Thus, the current study was conducted to determine if patients with hypothyroidism responded differently to L-T4 based on whether they carried the rs945006; T > G single nucleotide polymorphism (SNP) in the deiodinase type-3 gene. The current cross-sectional study was performed on 220 female Iraqi patients suffering from hypothyroidism. The patients were > 40 years old and were subjected to L-T4 therapy for a minimum of 120 days. The thyroid hormones (T4, T3, rT3, and T2) and stimulating thyroid hormones were tested for their levels in this study. The tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) was used for the genetic analysis, which allowed the detection of rs945006; T > G SNP. Regarding the genotypes distribution of rs945006, T > G SNP, the findings indicated that 11.4%, 22.7%, 65.9% were for the wild type, heterozygous mutants, and homozygous mutant type (GG), respectively. Based on their genetics, the patients were classified into three groups. The results showed that there were insignificant differences in the blood thyroid hormone levels of patients in different groups. As blood thyroid hormone levels are unrelated to the rs945006 SNP of the deiodinase type-3 gene, it has no impact on how well a patient responds to L-T4 therapy. However, given the homozygous mutant type is the most prevalent genotype among the subjects in the current investigation, the association between this SNP and hypothyroidism cannot be ignored. Since the DIO3 gene is predominantly expressed in the brain, more research is needed to determine the impact of this SNP on the thyroid hormone levels in the local brain tissue.

Keywords: Levothyroxine, rs945006, Iraqi patients, Deiodinase type-3 gene, Amplification Refractory Mutation System

Full length article **Corresponding Author*, e-mail: Suzanne.J@uokerbala.edu.iq

1. Introduction

A disease known as hypothyroidism is characterized by low levels of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) [1]. Neurological and musculoskeletal complaints, cardiovascular illness, and infertility may develop as a result of hypothyroidism that is either untreated or poorly managed [2]. Hypothyroidism affects up to 5% of the population, with a further 5% going untreated [3]. More than 99% of afflicted individuals have primary hypothyroidism. This condition is 8-9 times more likely to afflict women than men, and the incidence of primary hypothyroidism increases with age, reaching a peak between the ages of 30 and 50 [4]. Patients with hypothyroidism are diagnosed and followed up by performing thyroid function tests and clinical examinations [5]. The evaluation of thyroid-stimulating hormone (TSH, also known as thyrotropin) is widely regarded as the gold standard for monitoring thyroid health and responding to treatment [6]. In order to treat hypothyroidism, most patients are subjected to oral levothyroxine (L-T4), a synthetic form of T4 [7]. This treatment is performed to decrease clinical symptoms and normalize TSH, T4, and T3 levels [8]. For individuals with hypothyroidism, the standard replacement dose of L-T4 is 1.6 g/kg of body weight [9]. The L-T4 is transformed into the physiologically active hormone (T3) by type-1 and type-2 deiodinase enzymes [10]. Then again, the deiodinase type-3 (D3) converts T4 to reverse triiodothyronine (rT3) and T3 to diiodothyronine (T2). The rT3 and T2 have no biological activity. The inner ring deiodination is used by the D3 enzyme to accomplish this inactivation. T3 levels are subsequently altered inside the target cells [11]. In contrast to D2, T4, and T3 can positively regulate the D3 enzyme, increasing both T4 and T3 clearance as a result. D3 may therefore interfere with the signaling of local thyroid hormones, which might lead to systemic hypothyroidism [12]. When it comes to tissue protection against excessive thyroid hormone production in maturity, tissue development, and the embryonic phase in adulthood, deiodinase type-3 is crucial. The skin and central nervous system have the greatest amounts of it. When thyroid hormone overproduction poses a serious threat to life, it has been shown to have a protective effect in adults [11].

The deiodinase type-3 (DIO3) gene, found on human chromosome 14q32 [13], regulates the D3 enzyme

expression [13]. Abnormal phenotypes can be caused by mutations in the DIO3 gene, which alter D3 enzyme expression and/or activity [12]. The expression or stability of mRNA in the DIO3 gene may be affected by a single nucleotide polymorphism (SNP, 945006; T > G) in the 3' untranslated region [14].

There is widespread dissatisfaction with the inefficient therapeutic response of Iraqi hypothyroidism patients to the L-T4 drug. Even after months or years of treatment, these people continue to have hypothyroidism symptoms. The current study aimed to determine whether or not the rs459006; T > G SNP in the DIO3 gene influences the therapeutic response to L-T4 therapy. Regarding Iraqi hypothyroidism patients, this research is part of a larger effort to unveil how the deiodinase genes affect the way L-T4 works as a treatment for hypothyroidism.

2. Materials and methods

The Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala, Iraq, gave its approval to the current cross-sectional study with the reference number 2021HU6. Participants gave their informed consent after reading and understanding the study objectives. Also, patients were asked to fill out a questionnaire. Between November 2021 and March 2022, a total of 220 hypothyroid female patients were enrolled in this study. The participants were enlisted from primary hypothyroidism patients who were trying to schedule follow-up appointments at outpatient clinics. Hypothyroidism symptoms could be observed in all participants and no improvement was found after L-T4 treatment. All of the patients were locals of Kerbala province in Iraq. They were unrelated and aged ≥ 40 years. The subjects have been subjected to L-T4 treatment for a minimum of 120 days. Excluded from this study were individuals who were male or pregnant; who had a current or past neoplasm, autoimmune thyroid disease, or secondary hypothyroidism; who were using medications that could influence thyroid hormone bioavailability; or who were undergoing thyroidectomy. Participants who met the inclusion criteria had their blood drawn by a professional nurse. Blood (5 ml) was taken out and split in half. After 30 minutes, The first portion (3 mL) was centrifuged after being placed in a simple tube without an anticoagulant. The serum was separated and kept at a low temperature (-20 °C) for biochemical testing. The remaining blood (2 mL) was utilized for DNA extraction after being transferred to an evacuated ethylenediaminetetraacetic acid (EDTA) tube.

2.1. Genetic analysis

All of the blood samples had genomic DNA extracted using a China-made mini kit called Prep genomic DNA, following the manufacturer's instructions. Using the tetra primers amplification refractory mutation system-polymerase chain reaction (tetra ARMS-PCR) method, the DIO3 gene's rs459006; T > G SNP was identified. The primers were created with primer-BLAST software and bought as lyophilized materials from Alpha DNA Corporation, Canada (http://www.alphadna.com/contact.html). Each primer was precisely diluted in nuclease-free water until a stock solution with a concentration of 100 pmol/µl was obtained. The diluted working solution (10 pmol/µl) was created by diluting a stock solution of the outer forward, the outer reverse primers (10 µl), and 90 µl of nuclease-free water. The working solutions of the inner forward and the inner reverse primers (5 pmol/µl) consisted of 5 µl of their stock solutions and 95 µl of nuclease-free water. The primers sequences were as follows: the outer forward primer 5`TGGGTTCCAGGAGACTCTCAGCTCA3`, the outer reverse primer 5`GAGCACCCTCCCCCTCAAGGTTTA3`, the inner forward primer 5`TCCCTGGTAGGGGAAGTGATGTTGG3` and the inner reverse primer 5`GCCCACCCCTCCCCATTCA3`. The PCR reaction was performed with a total of 25 µL of PCR reaction mixture, including 11 µL of nuclease-free water, 1 µL (10 pmol/L) of each of the forward primers, 5 µL (100 ng/µL) of genomic DNA, and 1µL (5 pmol/L) of each the reverse primers, and 5 µL of Accu power PCR Pre Mix (Bioneer Company, Korea). the PCR program for the thermocycler (Veriti, ThermoFisher, USA) was initial denaturation for 3 minutes at 95°C, 30 cycles of amplification (denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 1 minute at 72°C), and final extension for 5 minutes at 72°C. The amplification was verified by electrophoresis using 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 mg/ml) [15,16].

2.2. Biochemical analysis

By employing competitive electrochemiluminescence immunoassay (ECLIA) kits from the Snibe Diagnostic Corporation, China, the *in vitro* quantitative measurement of thyrotropin, total and free T3, and total and free T4 was carried out. The catalog numbers of these kits were 130203001M, 130203003M, 130203005M, 130203002M, and 130203004M, respectively. T2, rT3, and fasting serum insulin were measured using the solid phase enzyme-linked immunosorbent assay (ELISA) method. The sandwich principle serves as the foundation for this method. The T2 and rT3 kits have the catalog codes EA0183Hu and EA0182Hu from BT Lab Corporation in China, respectively. The Mindray Corporation manufactured the insulin kit (China; catalog number 130205002M). A photometrical test was used to measure blood glucose using a lab kit (Mindray Company, China, with catalog number GLU0102). The amount of fasting serum glucose was measured at a wavelength of 510 nm.

2.3. Statistical analysis

The statistical program for the social sciences (SPSS) version 22 was used to examine the data that were acquired (SPSS Inc, Chicago, USA). For the purpose of comparing the means of the three research subject groups, a one-way analysis of variance (one-way ANOVA) was conducted. The distribution of alleles and genotypes in accordance with Hardy-Weinberg equilibrium was examined using the goodness of fit test, followed by a Chi-square analysis. The P value of 0.05 or less was regarded as statistically significant.

3. Results and Discussions

Thyroidologists commonly beilieve L-T4 monotherapy at doses that restore normal serum thyrotropin levels is sufficient to bring a patient to euthyroidism. This dogma is now in question due to data showing that L-T4 does not alleviate symptoms of hypothyroidism in a sizable percentage of patients. The symptoms include psychological [17] and metabolic [18] effects due to the problem in normalizing the serum levels of T3 in patients [19]. Since deiodinases enzymes (type-1 and type-2) vary in their performed activities, thyroxin cannot be normally transformed to T3. Moreover, different D3 enzyme activities prevent the normal metabolization of T4 into rT3. Consequently, the normal metabolization of T3 into T2 is prevented. Even with L-T4, these mechanisms might not be able to fully restore T4 and T3 to normal levels. Of note, the deiodinases expression in local tissues regulates the levels of thyroid hormones in certain regions. Consequently, the way thyroid hormone functions in different tissues depend on these levels [12].

Table 1 indicates the demographic data of 220 Iraqi females with primary hypothyroidism. To detect rs495006; T > G SNP in the DIO3 gene, Tetra ARMS-PCR was utilized. This technique involves the use of four primers, including two outer and two inner primers. When the G allele is present, the inner forward primer is devised to anneal, producing a band that appears at 186 bp on the agarose gel. When the inner reverse primer anneals, a band of 143 bp appears on the agarose gel, occurring in the presence of the T allele. The outside primers anneal in any scenario and offer a 285 bp "control" band. This indicates that the size of PCR products correlates with the existence of different alleles. As for the wild type (TT), two bands of 285 bp and 143 bp were obtained). In contrast to the homozygous mutant type (GG), which generated two bands of sizes 285bp and 186bp, the heterozygous mutant type (TG) produced three bands of sizes 285bp, 186bp, and 143bp, as shown in Figure 1.

Table 2 shows the distribution of the alleles and genotypes of rs945006; T > G SNP among the study participants. There was no discernible difference between the three groups with respect to age, body mass index, or length of treatment. As can be seen in Table 3, TSH, total and free T4, total and free T3, reverse T3, and T2 levels did not differ significantly across TT, TG, and GG carriers. Moreover, carriers of the three genotypes did not differ significantly from one another in terms of blood pressure measures and glycemic profile.

As far as the context of Iraq is concerned, the present research is the first genetic study examining how the rs945006; T > G SNP of the DIO3 gene affects TSH, L-T4, and thyroid hormone levels in L-T4-treated hypothyroid patients. The hypothesis was formed on the idea that SNPs in the DIO3 gene might influence D3 enzyme function. Consequently, this may have an impact on both the T4-to-rT3 and T3-to-2 conversions. This intern affects the hormone levels of T4 and T3.

Based on the obtained results, serum rT3, serum free and total T4, serum T2, serum TSH, serum free and total T3, and L-T4 dosage were not affected by the rs945006; T > G SNP (Table 3). These results agree with those of several prior genome-wide associated and candidate gene analyses [20-24]. Although this SNP had no effect on the patient's blood thyroid levels, it is of high significance to mention that DIO3 gene is highly expressed in the brain [11]. As a result, when compared to the blood, local tissue might experience variations in the amounts of thyroid hormones. Furthermore, the present research found that TT, TG, and GG did not differ significantly with regard to glycemic profile measures or blood pressure parameters (Table 3). Current patients with hypothyroidism who have been under L-T4 treatment for a minimum of 120 days may have genetic variants in other genes. Type-1 and type-2 deiodinase, the TSH receptor gene, and the genes encoding the proteins that transport T4 and T3 around the body are only some of the genes that might be examined to learn more about the mechanisms that control T4 and T3 metabolism.

DIO3 is also one of the imprinted genes; as a result, the way DIO3 polymorphism affects thyroid hormone homeostasis can be traced to the parental origin of the variant allele. Therefore, genomic imprinting may mitigate the impact of DIO3 polymorphisms on D3 enzyme function [25]. This may account for the fact that the rs945006; T > G SNP in the DIO3 gene was not significantly associated with any of the estimated thyroid, glycemic profile, or blood pressure parameters. Due to the widespread distribution of the mutant allele, it is possible that the DIO3 or rs945006; T > G SNP is related with hypothyroidism illness even if there is no substantial connection between this SNP and any of the serum thyroid hormones (G allele frequency = 0.77, Table 2). The majority of the 220 Iraqi hypothyroidism patients in the current investigation were homozygous mutants (65.9%). The DIO3 gene was previously studied in an animal model for its potential role in hypothyroidism. The findings showed that central hypothyroidism developed in the D3 deletion mouse model and persisted throughout the animal's lifespan [26]. This result supports the current hypothesis that DIO3 is involved in the onset of hypothyroidism.

Table 1. The demographic information of the hypothyroid patients

Parameters	Mean ± SD, (n= 220)
Age (year)	49.15 ± 9.11
BMI (Kg/m^2)	30.98 ± 5.83
Duration of the treatment (years)	4.47 ± 4.03

SD: Standard deviation, N: Number of studied subjects, BMI: Body mass index

Table 2. Participants' distribution of alleles and genotypes of rs945006; T > G

Genotype (N = 220)	Frequency (%)	Allele	Frequency	Chi-square	P- value
TT	25 (11.4)	T	0.23	26.947	0.0001
TG	50 (22.7)	G	0.77		
GG	145 (65.9)				

TT: Wild type, TG: Heterozygous mutant type, GG: Homozygous mutant type, N: Number of the study subjects

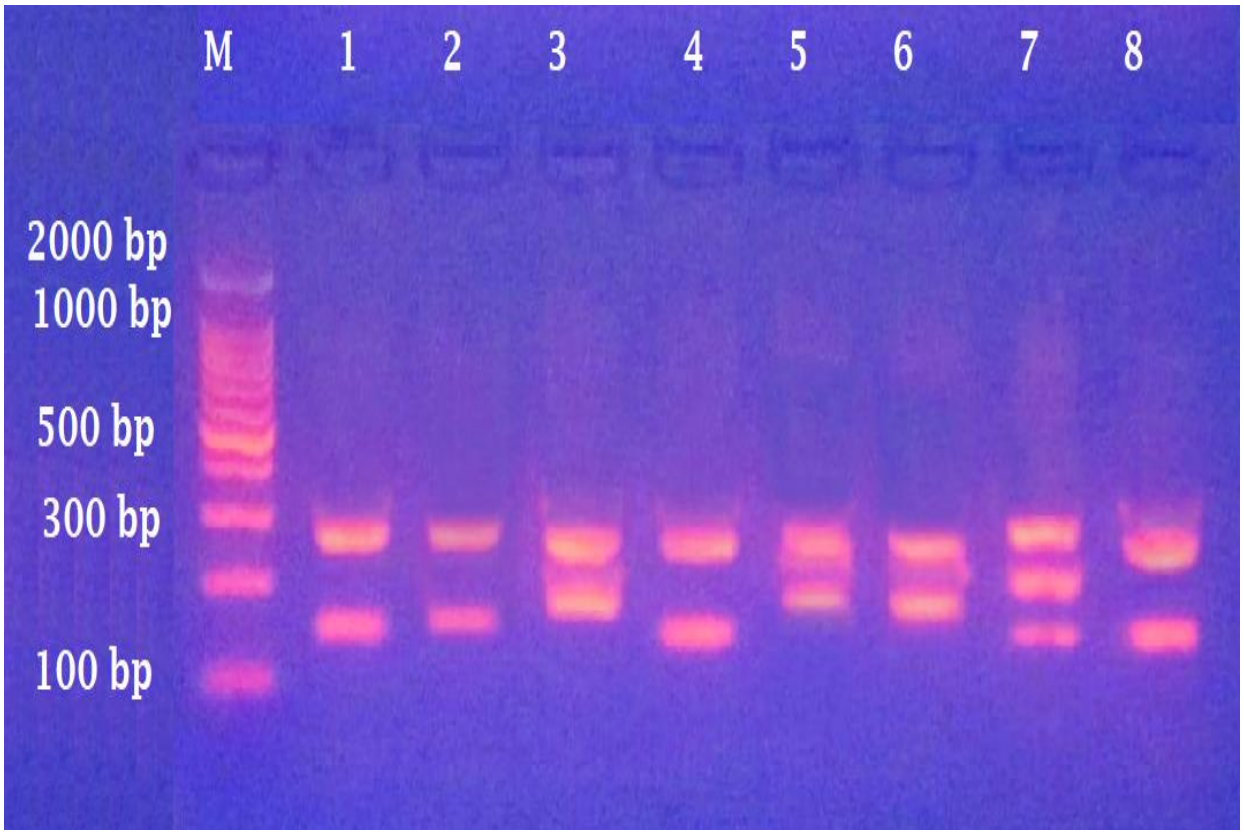

Figure 1. Polymerase chain reaction and horizontal agarose gel electrophoresis (1.5% w/v) to identify the rs945006 T > G SNP. M: 100 bp DNA marker. Wild type is shown by lanes 1, 2, 4, and 8, the heterozygous mutant type is shown by lane 7, and the homozygous mutant type is shown by lanes 3, 5, and 6

Table 3. The rs945006; T > G SNP genotypes in relation to the demographics, biochemical tests, and blood pressure parameters of Iraqi hypothyroidism patients

Parameter	Patients' genotypes, (N, =, 220)			P-value
	TT (N = 25)	TG (N = 50)	GG (N = 145)	
Age (year)	50.36 ± 1.24	50.04 ± 1.46	48.64 ± 0.75	0.51
BMI (Kg/m2)	29.08 ± 0.96	30.61 ± 0.93	31.44 ± 0.47	0.15
Duration of the treatment (years)	4.63 ± 0.87	4.33 ± 0.47	4.50 ± 0.35	0.94
Total T3 (nmol/L)	1.60 ± 0.09	1.54 ± 0.05	1.56 ± 0.03	0.86
Total T4 (nmol/L)	109.00 ± 7.18	100.41 ± 3.70	105.64 ± 2.62	0.45
Free T3 (pmol/L)	6.33 ± 0.25	6.44 ± 0.19	6.65 ± 0.13	0.51
Free T4 (pmol/L)	15.31 ± 1.01	15.18 ± 0.39	16.32 ± 0.66	0.53
TSH (µlU/L)	3.39 ± 0.94	4.99 ± 0.93	6.97 ± 1.07	0.24
rT3 (nmol/L)	0.93 ± 0.08	0.95 ± 0.05	0.92 ± 0.03	0.94
T2 (nmol/L)	2.00 ± 0.16	1.96 ± 0.15	2.06 ± 0.09	0.86
Total T3/ total T4	1.49 ± 0.11	1.59 ± 0.05	1.55 ± 0.04	0.70
fT3/fT4	0.44 ± 0.03	0.43 ± 0.01	0.43 ± 0.01	0.84
T3/rT3	1.86 ± 0.18	1.90 ± 0.12	1.93 ± 0.07	0.93
rT3/T4	0.93 ± 0.09	1.00 ± 0.07	0.94 ± 0.03	0.75
T4 dose (µg)	101.00 ± 7.96	85.00 ± 4.94	94.37 ± 3.56	0.22
FBS (mg/dL)	122.62 ± 9.00	108.05 ± 6.32	113.35 ± 4.20	0.47
Fasting insulin	12.39 ± 1.77	21.20 ± 3.43	16.63 ± 1.11	0.06
HOMA-IR	3.94 ± 0.62	5.54 ± 0.85	4.93 ± 0.47	0.49
Systolic BP (mmHg)	127.20 ± 3.08	127.20 ± 2.28	126.62 ± 1.27	0.96
Diastolic BP (mmHg)	75.20 ± 4.00	78.00 ± 2.13	78.82 ± 1.20	0.55
MAP (mmHg)	92.53 ± 2.29	94.40 ± 1.44	94.75 ± 0.82	0.60

The data is represented as mean ± standard error of the mean, N: Numbers of the study subjects, TT: Wild type, TG: Heterozygous mutant type, GG: Homozygous mutant type, T3: 3,3,5-Triiodothyronine, T4: Thyroxin, T2: 3,5-Diiodothyronine, rT3: Reverse triiodothyronine, FBS: Fasting blood sugar, FSI: Fasting serum insulin, HOMA-IR: Homeostatic model assessment for insulin resistance, BP: Blood pressure, MAP: Mean arterial pressure. The reference ranges of the hormones are as follows: Total T4 = 64.3-185 nmol/L, Total T3 = 0.92-2.33 nmol/L, Free T4 = 9-20 pmol/L, Free T3 = 2-9 pmol/L, TSH= 0.4-5 µlU/L.

4. Conclusions

In conclusion, rs945006; T > G SNP is unlikely to have any impact on L-T4 treatment because it has no influence on serum thyroid hormones and serum TSH levels. Nevertheless, in the current sample of Iraqi hypothyroidism patients, the homozygous mutant type (GG) is the most prevalent genotype, indicating that this SNP is associated with the condition.

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