



Deiodinase Type 3 Polymorphism (rs1190716) Affects Therapeutic Response to Levothyroxine

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ABSTRACT

Objectives: Levothyroxine (LT4) is a commonly used treatment for hypothyroidism. Deiodinase enzymes control the metabolism and homeostasis of thyroid hormones (THs). Deiodinase type 3 gene (*DIO3*) encodes deiodinase type 3 enzyme (D3), and the genetic polymorphisms of this gene could affect the levels of THs and the response to LT4 treatment. This study aimed to investigate the single-nucleotide polymorphism (SNP), rs1190716; C > T, of *DIO3* as a candidate genetic variant that might affect the clinical response to LT4 treatment.

Materials and Methods: Two hundred Iraqi hypothyroid female patients aged 40 years were enrolled in this cross-sectional study. All of them were already on the LT4 treatment for at least 4 months. THs [thyroxin (T4), triiodothyronine (T3), reverse triiodothyronine (rT3), and diiodothyronine (T2)] were estimated. An allele-specific polymerase chain reaction technique was performed to detect the rs1190716; C > T SNP.

Results: The genotypes distribution of rs1190716; C > T SNP was 10 (4.5%) for the wild type (CC), 50 (22.7%) for the heterozygous mutant type (TC), and 160 (72.7%) for the homozygous mutant type (TT). The patients were divided into three groups according to their genotypes. Significant differences were found in the T4, T3, and T2 levels among the patients ($p=0.019$, $p=0.039$, $p=0.032$, respectively).

Conclusion: The rs1190716; C > T SNP could affect the activity of the D3 enzyme and the metabolic homeostasis of the THs; therefore rs1190716; C > T SNP could have an impact on the therapeutic response to LT4 in Iraqi female patients with primary hypothyroidism. Regarding *DIO3* gene, this is a novel finding; hence, further studies are needed to confirm it.

Key words: Primary hypothyroidism, tetra ARMS-PCR, thyroxin, triiodothyronine, deiodinases type 3 enzyme

INTRODUCTION

Hypothyroidism is a common illness caused by the deficiency of thyroid hormones (THs).¹ The prevalence of hypothyroidism varies according to geographic region, population, and whether it is overt or subclinical hypothyroidism. Although it is roughly 3.2% overt hypothyroidism and 14.1% subclinical hypothyroidism in the Iraqi population.² It has been reported that the incidence of hypothyroidism is higher in females than in males and in older people, or at least the elderly, who receive hypothyroidism treatment more frequently.³

Levothyroxine (LT4) is a synthetic thyroxin (T4) that is suggested for the treatment of hypothyroidism and is physiologically and biochemically identical to the endogenous hormone. Treatment lessens hypothyroidism symptoms and

returns the thyroid stimulating hormone (TSH) and TH levels to a normal physiological range.⁴ Initial dose requirements for LT4 therapy can range significantly from small doses (25-50 µg) that are given to mild or subclinical cases to greater doses (88-175 µg) that are given to people who barely have any thyroid gland activity.⁵

Deiodinase enzymes are responsible for the metabolism of LT4. THs are activated and inactivated by deiodinases by removing iodine from the outer or inner ring of the THs molecules. Deiodinases enzymes are classified into three different types: type 1 (D1), type 2 (D2), and type 3 (D3). While type 3 enzyme inactivates T4 to reverse triiodothyronine (rT3) and T3 to diiodothyronine (T2), the first two enzymes convert T4 to triiodothyronine (T3).⁶ The primary deiodinase

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during embryonic development is D3, which also protects growing tissue from excessive TH synthesis at maturity. Under physiological conditions, it is mostly found in the skin and central nervous system and is absent or present in very small amounts in the other tissues.⁷

The deiodine type 3 gene (*DIO3*), which encodes D3 enzyme, is found in the imprinted region of the chromosomal locus 14q32.2. It is expressed from the human paternal allele preferentially during development.⁸ *DIO3* opposite strand upstream RNA (*DIO3OS*) gene, which overlaps with *DIO3* and is transcribed in the opposite direction, is located in same imprinting region but is dominantly expressed from a maternally inherited allele.⁹ *DIO3OS* gene is a long non-coding RNA that regulates the expression of a gene during overlap with a target gene or promoter or by modification of the chromatin structure during epigenetic marks. The human *DIO3OS* gene is widely expressed in many tissues. It has been reported that *DIO3OS* has the potential to control the expression and activity of *DIO3*.¹⁰ The *DIO3OS* gene contains six exons and two alternate polyadenylation sites. The genetic polymorphism rs1190716 is located in the intron region of this gene. Exonic sequence of *DIO3OS* overlaps with the *DIO3* promoter and strongly regulates its expression.¹¹

Many patients with hypothyroidism who received LT4 treatment complained that the disease symptoms persisted even after their serum TSH levels returned to normal.¹² This study is one of the studies series that investigated the impact of the deiodinases enzymes on the biochemical response to LT4 in Iraqi the effect of the rs1190716 variant of *DIO3* gene.

MATERIALS AND METHODS

Subjects and methodology

This cross-sectional study was approved by the Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala (reference number: 2021HU6).

Approximately 220 hypothyroid female patients were recruited from November, 2021 to March, 2022. Patients were recruited from outpatient clinics after they were diagnosed with primary hypothyroidism. The patients' age was 40 year old. They had already been on the treatment for at least 4 months. Patients that were not included in this study were male patients, pregnant patients, patients with an active neoplasm or a history of neoplasm, patients with a condition that may be the main cause of secondary hypothyroidism, patients who were taking drugs that may affect Th bioavailability, patients with autoimmune thyroid disease, and any patient who underwent thyroidectomy. All the participants were enrolled in this study after signing a written consent form that included a detailed explanation of the study purpose, and they were asked to fill out a specially designed questionnaire.

Blood (5 mL) was collected and divided into two parts, the first part (3 mL) was placed in an anticoagulant-free plain tube, and the second part (2 mL) was placed in an evacuated ethylenediaminetetraacetic acid (EDTA) tube. After 30 min of sampling, the blood sample in the plain tube was centrifuged,

and the serum was separated and stored at -20 °C for the biochemical analysis. For the DNA extraction, samples in EDTA tubes were used.

Genetic analysis

The genomic DNA was isolated from the whole blood samples using a Prep genomic DNA Mini kit (China) based on the procedure recommended by the manufacturer. To detect the rs1190716 single-nucleotide polymorphism (SNP), the tetra primers amplification refractory mutation system-PCR (tetra ARMS-PCR) technique was used. The primers were designed using primer-BLAST software and were purchased as lyophilized products from Alpha DNA, Canada. Each primer was dissolved in specific volumes of nuclease-free water to obtain a stock solution with a concentration of 100 pmol/μL. Diluted work solutions were prepared by adding 90 μL of nuclease-free water to 10 μL of each stock solution of the outer forward and outer reverse primers. Water (95 μL) was mixed with 5 μL of the stock solution of each of the inner forward and inner reverse primers to make the work solution of each primer. The work solutions were kept at -20 °C until use. The sequences of primers are as outer forward primer 5'ATCCCTGGGGCTAGAAAGAG3', the reverse primer 5'TTTTCCCACAGTTAGTTTCAGAAA3', the inner forward 5'CCACCCAGACCATACTTGCT3', the inner reverse g'CGCATGTGCATGTGTTTG3'. The PCR reaction mixture volume was total 25 μL, it contained 5 μL of Accu power PCR Pre Mix (Bioneer Company, Korea), 5 μL (100 ng/μL) of the extracted DNA, 1 μL (10 pmol/L) of each of the forward primers, 1 μL (5 pmol/μL) of each of the reverse primers and 11 μL of nuclease-free water. The program used for the amplification was as follows: initial denaturation for 3 min at 95 °C, 30 cycles of amplification (denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 60 °C, extension for 1 min at 72 °C), and a final extension for 5 min at 72 °C. To confirm the amplification and detect the PCR products, electrophoresis using agarose gel [1.5% (w/v)] was performed.

Biochemical analysis

The competitive electrochemiluminescence immunoassay (ECLIA) was used to determine the levels of TSH, total T3 (tT3), free T3 (fT3), total T4 (tT4), and free T4 (fT4). Immunoassays were performed using laboratory kits (Snibe Diagnostics, China). A solid phase enzyme-linked immunosorbent assay (ELISA) was used to determine T2, rT3, and fasting serum insulin. T2 and rT3 analyzes were performed using research kits from BT LAB, China, while fasting serum insulin levels were estimated using a diagnostic kit from Mindray Company, China. A photometric assay method was used to determine the serum glucose concentration, which was estimated at a wavelength of 510 nm. This assay was performed using a diagnostic kit from Mindray Company, China.

Statistical analysis

To analyze the data, the statistical package for social sciences (SPSS) software (version 22, Chicago, USA) was used. The means of the three study groups were compared using one-way ANOVA. A *post-hoc* test was conducted to assess the

multiple comparisons between the means of the groups. The chi-squared goodness of fit test was performed to evaluate the allele distribution in accordance with Hardy-Weinberg equilibrium. The difference between the groups was considered significant when $p < 0.05$.

RESULTS

Two hundred twenty Iraqi females with primary hypothyroidism were enrolled in this study. Table 1 demonstrates the demographic characteristics of the studied patients.

To detect the rs1190716; C > T SNP, tetra-ARMS-PCR was performed. The PCR amplicon sizes differ according to the presence of C or T alleles. In case of the wild type (CC), two PCR bands with 253 and 121 bp appeared on the agarose gel. In mutant (CT), three PCR bands with 253 bp, 170 bp, and 121 bp appeared, whereas two PCR bands with 253 bp and 170 bp appeared in the case of the homozygous mutant type (TT), as demonstrated in Figure 1.

Table 2 demonstrates the allele and genotype distribution of the rs1190716; C > T SNP.

The age and body mass index did not show significant differences among the three groups of patients, but the duration

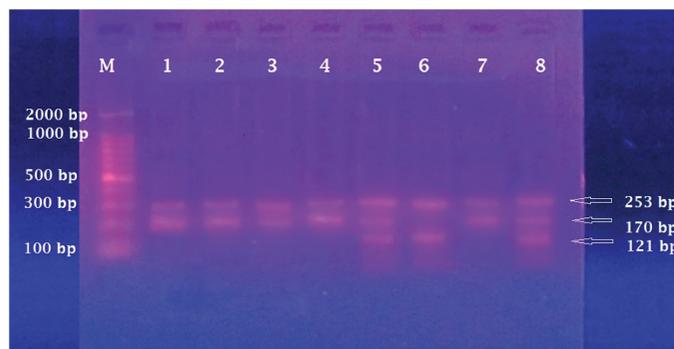


Figure 1. The agarose gel electrophoresis for identifying rs1190716; C > T SNP using tetra ARMS-PCR. M: DNA ladder, lanes 1, 2, 3, 4, and 7 represent the homozygous mutant type (TT), lanes 5 and 8 represent the heterozygous mutant type (CT), and lane 6 shows the wild type (CC)

Table 1. The characterization of the hypothyroidism patients

Parameters	Mean \pm SD (n: 220)
Age (year)	49.15 \pm 9.11
BMI (kg/m ²)	30.98 \pm 5.83
Treatment duration (years)	4.47 \pm 4.03

n: Number of studied subject, SD: Standard deviation, BMI: Body mass index

Table 2. The allele distribution of rs1190716; C > T SNP in the hypothyroidism patients

Genotype (n: 220)	Frequency (%)	Allele	Frequency	Chi-square	p value
CC (wild type)	10 (4.5)	C	0.16	4.94	0.084
CT (heterozygous mutant type)	50 (22.7)	T	0.84		
TT (homozygous mutant type)	160 (72.7)				

n: Numbers of the study subjects, SNP: Single-nucleotide polymorphism

of treatment was significantly different. The levels of TSH, fT4, fT3, and rT3 did not display significant differences among the groups of patients; however, there was a significant difference in tT3 level between CT and TT groups. A significant difference was also detected in T2 level between the same groups. The tT4 levels showed significant differences between CC and TT carriers (Table 3).

No significant differences were obtained in the blood pressure (systolic pressure, diastolic pressure, and the mean arterial pressure) among the groups of patients or in the glycemic indices (Table 3).

DISCUSSION

The rationale for LT4 replacement treatment is that two deiodinases, D1 and D2, convert T4 to T3 (the active form hormone), whereas D3 clears T4 to rT3 and T3 to T2. These three pathways restore the pool of T3 and the clinical euthyroidism.¹³ After the observation of many patients, who were treated with the LT4, high serum T4/T3 levels were detected, and the symptoms of hypothyroidism have not resolved. However, the level of TSH was within the normal reference range. Thus, LT4 to restore TH levels came into question.¹⁴ This could be the first study in Iraq and the Middle East that searches the impact of the rs1190716; C > T SNP on the clinical response to LT4 among patients with hypothyroidism. This response can be assessed by estimating the serum TSH and TH levels and the LT4 dose. We hypothesize that the SNPs in *DIO3* gene, or other genes that overlap with it, such as *DIO3OS*, might impact on the activity of D3 enzyme. Accordingly, the conversion of T4 and T3 to rT3 and T2, respectively, might be affected, which affects the levels of T4 and T3 hormones.

This study indicated that there was no significant association between rs1190716; C > T SNP and TSH level, fT3, fT4, rT3, and LT4 dose. tT4, tT3, and T2 were significantly different among the three groups of patients. The patients with mutant allele (T) had significantly lower tT4 and greater tT3 levels; at the same time, they had slightly higher TSH levels and slightly higher LT4 dose (Table 3). The patient group carrying the wild-type allele had significantly higher T4 levels, slightly lower LT4 doses, and lower TSH levels. This could be due to the role of D2, which, as an enzyme inhibited by its substrate, D2 activity can be inhibited by the high T4 concentration. This results in decreasing T3 concentration, whereas serum TSH concentration could be within the normal range because of the slightly elevated T4 concentration.¹⁵ According to a study conducted on rats, the hypothalamic tancytes and pituitary thyrotrophs absorb plasma T4 and locally convert it to T3 by

Table 3. The demographic, the thyroid function, the blood pressure, and the glycemic analysis of the study subjects

Parameter	Genotypes (n: 220)			p value
	CC (n: 10)	CT (n: 50)	TT (n: 160)	
Age (year)	51.20 ± 2.96	47.98 ± 1.17	49.39 ± 0.73	0.48
BMI (kg/m ²)	29.55 ± 1.40	31.16 ± 0.65	31.02 ± 0.49	0.72
Duration of the treatment (years)	7.63 ± 1.83	4.38 ± 0.46	4.30 ± 0.32	0.04 ^{*a,b}
tT3 (nmol/L)	1.49 ± 0.10	1.71 ± 0.07	1.52 ± 0.03	0.019 ^{*c}
tT4 (nmol/L)	125.74 ± 9.52	108.81 ± 5.31	102.29 ± 2.22	0.039 ^{*b}
fT3 (pmol/L)	6.40 ± 0.41	6.60 ± 0.28	6.56 ± 0.10	0.93
fT4 (pmol/L)	16.54 ± 1.47	15.65 ± 0.74	16.00 ± 0.58	0.91
TSH (μIU/mL)	3.03 ± 0.90	7.29 ± 2.20	5.94 ± 0.76	0.51
rT3 (nmol/L)	0.86 ± 0.10	1.01 ± 0.05	0.91 ± 0.02	0.21
T2 (nmol/L)	1.97 ± 0.30	2.38 ± 0.20	1.92 ± 0.07	0.032 ^{*c}
tT3/tT4	1.22 ± 0.09	1.61 ± 0.07	1.56 ± 0.03	0.06
fT3/fT4	0.40 ± 0.03	0.44 ± 0.01	0.43 ± 0.009	0.57
T2/tT3	1.32 ± 0.18	1.54 ± 0.13	1.31 ± 0.05	0.54
rT3/T4	0.72 ± 0.10	1.01 ± 0.06	0.95 ± 0.03	0.19
T4 dose (μg)	67.50 ± 9.16	97.00 ± 5.23	93.34 ± 3.35	0.11
FBS (mg/dL)	123.14 ± 16.82	121.49 ± 9.50	109.99 ± 3.23	0.27
Fasting insulin	24.24 ± 8.75	20.16 ± 2.93	15.82 ± 1.06	0.09
HOMA-IR	6.07 ± 1.61	6.18 ± 1.06	4.50 ± 0.37	0.13
Systolic BP (mmHg)	124.00 ± 3.05	126.60 ± 2.36	127.06 ± 1.21	0.82
Diastolic BP (mmHg)	80.00 ± 1.49	78.20 ± 2.17	78.12 ± 1.25	0.93
MAP (mmHg)	94.66 ± 1.87	94.33 ± 1.44	94.43 ± 0.82	0.99

Post-hoc test: a: CC vs. CT, b: CC vs. TT, c: CT vs. TT

The data is represented as mean ± standard error of the mean, *Significant at $p > 0.05$. n: Numbers of the study subjects, T3: 3,3,5-triiodothyronine, T4: Thyroxine, 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

D2. TSH secretion is decreased because of the D2 action at these two locations.¹⁴

Although D3 enzyme is not implicated in the T4 to T3 conversion, *DIO3* gene is also not involved. It is worthy to mention that there are other candidate genes that could be potential effectors. Deiodinase type 1 (DIO1), deiodinase type 2 (DIO2), and Th transporters notably influence the response to LT4. Previous studies suggested that common genetic variations in these loci might have an impact on the changes in the levels of TH metabolites and, accordingly, the response to LT4 treatment.^{16,17}

While normalizing serum TSH is the aim of LT4 therapy, a gradual increase in LT4 dose raises the circulating T3 levels while concurrently lowering TSH secretion (predominantly through D2).¹⁸ Our patients, who had the mutant allele, had a higher LT4 dose but lower tT4 and higher TSH levels (Table 3). This indicated that LT4 treatment did not restore normal TSH levels, which could be explained by the carriers of the mutant

allele (CT and TT) having lower tT4 levels. Thus, their TSH levels were above the reference range, so they needed a higher LT4 dose. This could be an indication that the rs1190716; C > T SNP increases D3 enzyme activity, which means increasing the conversion of T4 to rT3, thereby making the levels of tT4 lower in the mutant allele carriers. It was confirmed by the increased rT3 levels in the mutant allele carriers compared with the wild allele carriers, but not to a significant level (Table 3). As a result, the ratio of rT3/T4 was increased in the mutant types (CT and TT) groups but also in a non-significant manner (Table 3).

TSH level was lower (within the normal range) in the wild-type group than in both hetero and homo mutant type carriers, but not to a significant level (Table 3). This was due to the feedback inhibition of the elevated level of tT4 in this group. At the same time, this could indicate that the hypothyroidism patients, who were not carriers for the rs1190716; C > T SNP might have a better response to LT4 therapy and that D3 enzyme could have a role in TH regulation.

Our findings could be novel regarding rs1190716; C > T SNP and *DIO3* gene because previous studies demonstrated that this SNP and this gene had no impact on TH and TSH levels.¹⁹⁻²¹

The results also demonstrated that no significant differences in the glycemic parameters or blood pressure parameters were found among the three groups of patients. This role the rs1190716; C > T SNP from being involved as a risk factor for these parameters.

Study limitations

This study assessed the response to LT4 replacement therapy among the hypothyroidism patients by estimating serum Th levels. Deiodinase type 3 enzyme activity should be estimated in future studies to confirm the role of this enzyme.

CONCLUSION

The mutant allele (T) was the most frequent allele in our study subjects. The rs1190716; C > T SNP was significantly associated with the levels of tT3, tT4, and T2. Accordingly, SNP could affect the activity of the D3 enzyme and the metabolic homeostasis of the THs; therefore, rs1190716; C > T SNP could have an impact on the therapeutic response to LT4 in Iraqi female patients with primary hypothyroidism. This is a novel finding regarding *DIO3* gene; hence, further studies are needed to confirm it.

Ethics

Ethics Committee Approval: This cross-sectional study was approved by the Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala. The reference number for scientific and ethical approval is 2021HU6.

Informed Consent: Written permission was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.H.M., Concept: B.H., S.J., Design: B.H., S.J., Data Collection or Processing: A.H.M., B.H., S.J., Analysis or Interpretation: A.H.M., B.H., S.J., Literature Search: A.H.M., Writing: A.H.M., B.H., S.J.

Conflict of Interest: No conflict of interest was declared by the authors.

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