

Deiodinase Type III Polymorphism (Rs1190716) Affects The Therapeutic Response to Levothyroxine
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ABSTRACT

Objectives: Levothyroxine (LT4) is the commonly used treatment for hypothyroidism. Deiodinases enzymes control the metabolism and homeostasis of thyroid hormones (THs). Deiodinases type III (DIO3) gene encodes deiodinase type 3 enzyme (D3), the genetic polymorphisms of this gene could affect the levels of THs and then the response to LT4 treatment. This study aims 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 who were aged 40 years or older were enrolled in this cross-sectional study. All of them were already on the LT4 treatment for at least 4 months. Thyroid hormones (thyroxin (T4), triiodothyronine (T3), reverse triiodothyronine (rT3) and diiodothyronine (T2)) were estimated. 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 levels of T4, T3 and T2 among the groups of 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 in the therapeutic response to LT4 in Iraqi female patients with primary hypothyroidism. Regarding the DIO3 gene, this is a novel finding, hence further studies are needed to conform it.

Keywords: 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 the 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 is reported that the incidence of hypothyroidism is higher in females than in males, and it is higher in older people, or at least the elderly receive hypothyroidism treatment more frequently⁽³⁾.

Levothyroxine (LT4) is a synthetic thyroxin (T4) that is suggested for the treatment of hypothyroidism, and it is physiologically and biochemically identical to the endogenous hormone. Treatment aims to lessen hypothyroidism symptoms and return the thyroid stimulating hormone (TSH) and THs levels to a normal physiological range⁽⁴⁾.

Initial dose requirements for levothyroxine therapy can range significantly from small doses (25–50 µg) that are given to the mild or to the subclinical cases to greater doses (88–175 µg) that are given to the people who barely have any thyroid gland activity⁽⁵⁾.

Deiodinase enzymes are responsible for the metabolism of LT4. Thyroid hormones are activated and inactivated by deiodinases through removing an iodine from either the outer or the inner ring of the THs molecules. Deiodinases enzymes come 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 during embryonic development is D3, which also protects growing

tissue from excessive THs synthesis in maturity. Under physiological conditions, it is mostly found in the skin and central nervous system and it is absent or present in very little amounts in the other tissues ⁽⁷⁾.

The deiodinase type III gene (DIO3), which encodes the D3 enzyme, is found in the imprinted region of the chromosomal locus 14q32.2. It is expressed from the human paternal allele in a preferential manner during the development ⁽⁸⁾. The 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 dominantly expressed from a maternally inherited allele⁽⁹⁾. DIO3OS gene is long noncoding RNA regulates expression of gene during overlap with target gene or promoter, or by modification of chromatin structure during epigenetic marks. The human DIO3OS gene is widely expressed in many tissues. It is 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 on the intron region of this gene. Exonic sequence of a DIO3OS overlaps with the DIO3 promoter and strongly regulates its expression ⁽¹¹⁾.

Many hypothyroidism patients 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 studies series that search the impact of the deiodinases enzymes on the biochemical response to LT4 in Iraqi hypothyroidism female patients, it investigates the effect of rs1190716 variant of DIO3 gene.

Subjects and Methodology:

This is a cross-sectional study that was approved by the Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala, the reference number of the scientific and ethical approval is 2021HU6. About 220 hypothyroid female patients were recruited from November 2021 to March 2022. Patients were recruited from outpatient clinics after they have already been diagnosed with primary hypothyroidism. The patients' age was 40 years or older. They have already been on the treatment of LT4 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 the thyroid hormone bioavailability, patients with autoimmune thyroid disease, and any patient 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 requested to fill a specially designed questionnaire.

Blood (5 ml) was collected and divided into two parts, the first part (3 mL) was put into an anticoagulant-free plain tube and the second part (2 mL) was put into an evacuated ethylenediaminetetraacetic acid (EDTA) tube. After 30 min of sampling, the blood sample that is in the plain tube was centrifuged, then the serum was separated and stored at -20 °C for the biochemical analysis. For the DNA extraction, the samples in the EDTA tubes were utilized.

Genetic analysis

The genomic DNA was isolated from the whole blood samples using Prep genomic DNA Mini kit, China based on the procedure that recommended by the manufacturer. To detect rs1190716 single nucleotide polymorphism (SNP), tetra primers amplification refractory mutation system-PCR (tetra ARMS-PCR) technique was used. The primers were designed using primer-BLAST software and 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 was made by adding 90μl of nuclease-free water to 10 μl of each of the stock solution of the outer forward and the outer reverse primers. Water (95 μl) was mixed with 5 μl of the stock solution of each of the inner forward and the inner reverse primers to make the work solution of each one of them. The work solutions were kept at -20°C until they were used. The sequences of primers are as the following: the outer forward primer 5' ATCCCTGGGGCTAGAAAGAG3', the outer reverse primer 5' TTTTCCCACAGTTAGTTTCAGAAA3', the inner forward 5' CCACCCAGACCATACTTGCT3', the inner reverse g' CGCATGTGCATGTGTTTG3'. The PCR reaction mixture volume was totally 25 μL, it contained 5μL of Accu power PCR Pre Mix (Bioneer company, Korea), 5 μL (100ng/ μL) of the extracted DNA, 1 μL (10 pmol/L) of each of the forward primers, 1μL (5 pmol/μL) of each the reverse primers and 11 μL of nuclease-free water. The program that was used for the amplification is as follows: 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 a final extension for 5 minutes at 72°C. To prove the amplification and detecting the PCR products, electrophoresis using agarose gel (1.5% (w/v)) was performed.

The biochemical analysis

The competitive electrochemiluminescence immunoassay (ECLIA) was used to determine the level TSH, total T3 (tT3), free T3 (fT3), total T4 (tT4) and free T4 (fT4). This immunoassay was performed using laboratory kits (Snibe Diagnostic, China). A solid phase enzyme-linked immunosorbent assay (ELISA) was used to determine T2, rT3 and fasting serum insulin. The T2, rT3 analysis were performed using research kits from BT LAB, China, while the

fasting serum insulin was estimated using a diagnostic kit from Mindray Company, China. A photometrical assay method was used to determine the serum glucose concentration, which was estimated at the wave length of 510 nm, this assay was done 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 analysis of variance (one-way ANOVA). A post hoc test was conducted to assess the multiple comparisons between the means of the groups. The Chi-square from goodness of fit test was performed to evaluate the allele distribution in accordance to 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 characteristic of studied patients.

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

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

The age and BMI did not show significant differences among the three groups of the patients, but the duration of treatment was significantly different. The levels of the TSH, the fT4, the fT3 and the rT3 did not show significant differences among the groups of patients, however there was a significant difference in tT3 level between CT group and TT group. A significant difference was also detected in T2 level between the same groups. The tT4 levels show significant difference 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 nor obtained in the glycemic indices (Table 3).

Discussion

The rationale of 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 treated with the LT4, high serum T4/T3 was detected besides that the symptoms of hypothyroidism have not resolved although the level of TSH was within the normal reference range. Thus the ability of the LT4 to restore THs levels became into question⁽¹⁴⁾.

This could be the first study in Iraq and the Middle East area that searches the impact of the rs1190716; C>T SNP on the clinical response to LT4 among the hypothyroidism patients. This response can be assessed by estimating the serum TSH and the THs level in addition to the LT4 dose. Our hypothesis is that the SNPs in the DIO3 gene, or other genes that overlap with it such as DIO3OS, might have an impact on the activity of the D3 enzyme. Accordingly the conversion of T4 and T3 to rT3 and T2, respectively might be affected, which intern affect 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. While tT4, tT3, and T2 were found to be significantly different among the three groups of patients. The patients with mutant allele (T) had significantly lower tT4 and greater tT3 levels, in the same time they had slightly higher TSH levels and slightly higher LT4 dose (Table 3). While the patients group who carried the wild type allele had significantly higher T4 level, slightly lower LT4 dose and lower TSH level. 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, meanwhile serum TSH concentration could be within the normal range because of the slightly elevated T4 concentration⁽¹⁵⁾. According to a study carried out on rats, the hypothalamus tanycytes and pituitary thyrotrophs absorb plasma T4 and locally convert it to T3 by the D2, thus TSH secretion is decreased as a result of the D2 action at these two locations.⁽¹⁴⁾

Although the D3 enzyme is not implicated in the T4 to T3 conversion, therefore DIO3 gene is also not involved, it is worthy to mention that there are other candidate genes to be potential effectors. Deiodinase type I (DIO1), deiodinase type II (DIO2) and thyroid hormone transporters could notably influence the response to LT4. Previous studies suggested that common genetic variations in these loci might have an impact on the changing of 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 higher LT4 dose but lower tT4 and higher TSH levels (Table 3). This indicates that LT4 treatment did not

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

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

Our findings could be novel regarding the rs1190716; C>T SNP and DIO3 gene because the previous studies demonstrated that this SNP and this gene had no impact on the THs and TSH levels^(19, 20, 21).

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

Study Limitations

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

Conclusion

The mutant allele (T) is 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, this 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 in 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 conform it.

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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

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, N: Numbers of the study subjects, 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, *: Significant at P>0.05.

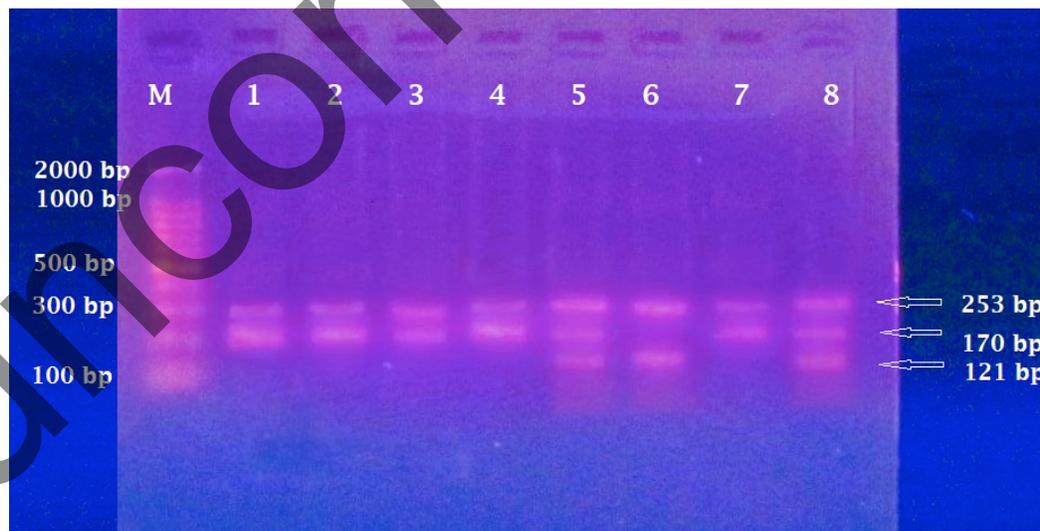


Figure 1: The agarose gel electrophoresis for identifying rs1190716; C>T SNP using tetra ARMS-PCR. M: DNA ladder, lanes 1, 2, 3, 4, and 7 represent the homozygous mutant type (TT), lanes 5 and 8 represent the heterozygous mutant type (CT), and lane 6 shows the wild type (CC).

Uncorrected proof