

The Association of *TSHR* Gene rs2268458 Polymorphism with Hypothyroidism in Females of Babylon Province-Iraq

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Abstract

The Aim: To evaluate the association of *TSHR* gene polymorphism rs2268458 with some Physio-biochemical parameters among hypothyroidism women.

Methods: This study included 51 hypothyroidism women and 43 healthy women as a control group. Some Physio-biochemical (body mass index (BMI), cytochrome 450 1A1 (CYP1A1), calcium, and phosphorus) and hormonal assay (Osteopontin (OPN), thyroid stimulating hormone (TSH), fT3, fT4 and Calcitonin (CT)) assay were performed. Genotyping of rs-2268458 of *TSHR* gene was carried out using the polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) technique.

Results: *TSHR* rs2268458 was associated with hypothyroidism in Iraqi women and allele C can be a risk factor for disease by OR= 4.393, CI=1.42-13.53 where TC and CC genotypes were associated with high BMI, while TC genotype was associated with high calcitonin and CYP1A1 levels.

Conclusion: The *TSHR* rs2268458 polymorphism was associated with hypothyroidism in Iraqi women and allele C can be a risk factor for some Physio-biochemical and hormonal disorder in hypothyroidism women.

1- Introduction

Hypothyroidism is an endocrine disorder defined as a deficiency in thyroid hormone production [1]. It is linked with a variety of signs as fatigue, bradycardia, depression, obesity, and hyperlipidemia [1],[2]. Thyroid hormones act a crucial physiological action on the metabolism and individual development [3]. The thyroid hormone receptor act as a transcription factors ligand to block or initiate the expression of a target gene after binding to thyroid hormone response elements (TRE) in promoter regions of the target gene[4]. When thyroid hormone is absent, thyroid receptors inhibit linked genes expression [5]. Thyrotropin or thyroid stimulating hormone (TSH) is the mainly Sensitive and valuable test for thyroid function [1]. TSH regulates the secretion of the thyroid gland and growth through binding to its receptors (TSHR) [6]. Genetic variants in the *TSHR* gene was described to be associated with the risk of thyroid diseases. [7]

The previous genome-wide association studies showed that chromosome 14 has a high level of association with hypothyroidism disease and the highest region are in 35Mbp, region 95Mbp and the highest region in the site of 93Mbp is near to the polymorphism (rs 2268458) which locate at region 81Mbp as show in the figure (1).

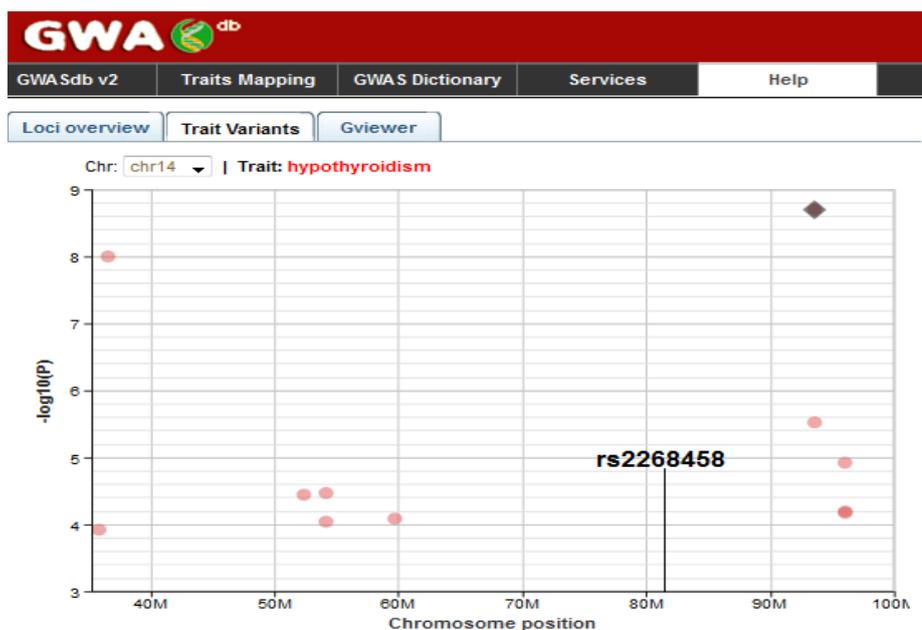


Figure (1): The association of chromosome 14 with hypothyroidism according to genome-wide association studies plotted by GWASdb.

Polymorphism in *TSHR* rs2268458 which located in intron 1 of the *TSHR* gene had been confirmed its association with Graves' Disease (GD) in Caucasians[8]. So this study planned to evaluate the association of *TSHR* gene polymorphism rs2268458 with the risk and the prognosis of hypothyroidism in Iraqi women (Babylon Province).

Subjects and Methods

The samples were collected from Mrjan teaching hospital/ Babylon province/ Iraq and patients were diagnosed by a physician which included 51 hypothyroidism women and 43 healthy women. Blood was withdrawn (5 ml) and dispersed into 2 parts, the first part in the anticoagulant-free plain tube for biochemical assays and the second part (2 ml) into an EDTA tube for DNA extraction. Centrifugation of blood had been made after 30 minutes of sampling and storage of serum was at -20°C until the biochemical assays (CYP1A1, calcium, and phosphorus) and hormonal assays (OPN, TSH, fT3, fT4, and CT) were performed. All routine study parameters were estimated in serum samples of these 51 hypothyroidism women and healthy control women using standard techniques. Levels of TSH (Bio-merieuxSA/ France), fT3, fT4, OPN, CYP1A1, and calcitonin (Elabscience) were evaluated by Enzyme-Linked-Immuno-Sorbent Assay (EL-ISA).

Calcium (Randox/ United Kingdom) and phosphorus (Spinreact, S.A.U./SPAIN) levels were done by a colorimetric method and the absorbance of color was read by spectrophotometer at 570 nm for calcium and 710 nm for phosphorus. The formula $\text{BMI} = \text{weight (kg)} / \text{height}^2 \text{ (m)}^2$ was used for estimation of body mass index (BMI).

Exclusion criteria

There are several pathological, normal physiological conditions and daily habit can interfere with hypothyroidism case were excluded from this study such as heart disease, hypertension, diabetes mellitus, pregnancy, and smokers.

DNA extraction

Genomic DNA from white blood cells (WBCs) for both hypothyroidism patients and control group were extracted by using DNA extraction kit (Favorgen/Taiwan) according to the standard protocol provided by the company.

PCR-RFLP

Amplification of the genomic DNA was by the following steps: denaturation at 94°C for 5 minutes, DNA amplification using 30 cycles. Each cycle contains the subsequent steps: denaturation at 94°C for the 30s, annealing for 30s, extension at 72°C for 40s, final elongation at 72°C for 7 minutes. The amplified DNA was electrophoresed on 2% agarose with ethidium bromide, (1X) TBE buffer at 100 V for 60 minutes. A 100 bp ladder was used as a marker for estimating the size of the fragment.

The digestion with restriction enzyme was performed in 50 µl containing 1µl of *ALUI* restriction enzyme (Biolabs/England), 10 µl of PCR product, 5 µl of enzyme buffer (1x) and the volume was completed with nuclease deionized water and incubated at 37°C overnight. Products of PCR were digested fragments and detected by electrophoresis on 8 % polyacrylamide gel.

Table 1: Sequences of primers used for PCR. .amplification of microRNAs genes

Gene name	Primer sequence	Reference
<i>TSHR</i> gene	F: 5'-CTAACCAGCAGAGGGAGCAC3' R: 5'-CCACTGCTTAAAGCCCAGAT3'	(Dechairo <i>et al.</i> , 2005) (Yin <i>et al.</i> , 2008)

Statistical Analysis

Analysis of the data was by using completely randomized design (CRD), Odd ratio and Pearson's goodness of fit chi-square test. The p-value ≤ 0.05

was regarded as significant. All analyses were performed using software of SPSS for Windows version 19.

Results

DNA Extraction

The result of DNA extraction was revealed in the figure (2).

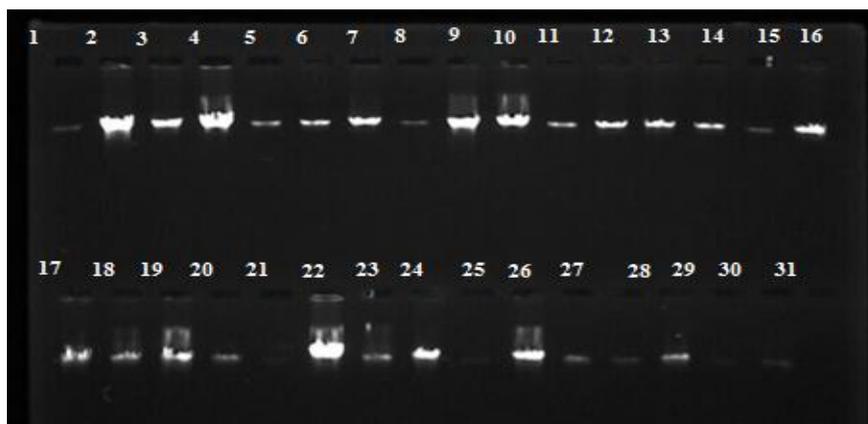


Figure (2): The electrophoresis pattern of genomic DNA extracted from WBC of hypothyroidism and healthy women, lane (1-16) for control and lane (17-31) for hypothyroidism women, 1% agarose, 75 V, 20 Am for 1 hour.

Determination of Annealing Temperature of *TSHR* Gene rs2268458 (Gradient PCR).

Annealing temperature had been determined by using temperature gradient (66-69°C) and the optimum annealing temperature was 69°C as shown in Figure (3).

Genotyping of *TSHR* rs 2268458

The results of *TSHR* gene amplification showed that the amplification product was 162 bp for patients and control as explained in the figure (4).

The product was digested with a restriction enzyme which cut the PCR- amplicon if C allele present resulting in 100 and 62 bp fragments, by the other hand if T allele present the amplicon will not be cut and appear as intact 162 bp as shown in figure (5).

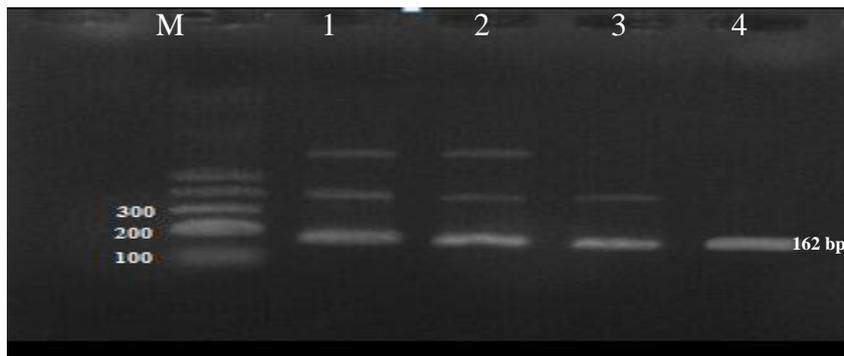


Figure (3): The electrophoresis pattern of gradient PCR product of *TSHR* gene at the different annealing temperature, the optimum annealing temperature was 69°C, lane M: DNA ladder, Lane 1: 66°C, Lane 2: 67°C, Lane 3: 68°C, Lane 4: 69°C.

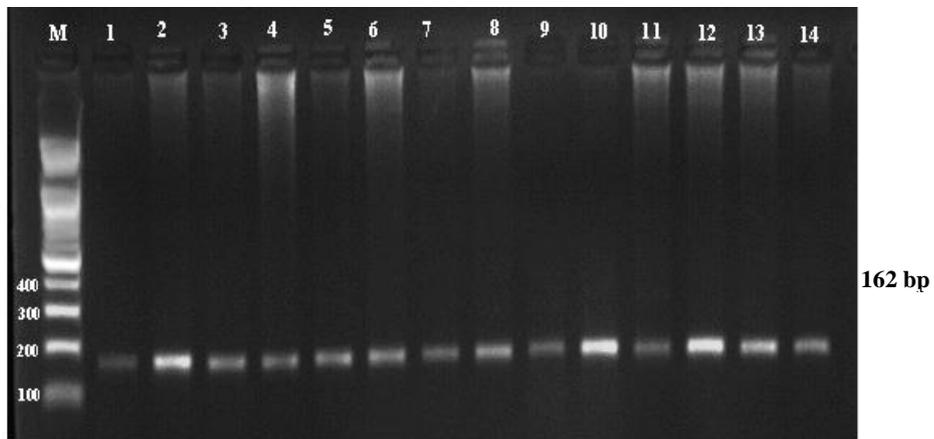


Figure (4): Electrophoresis pattern of PCR product for *TSHR* gene, amplification product was one band 162 bp, lane M: DNA ladder, lane (1-9): PCR product of patients, lane (10-14): PCR product of healthy subjects, 1% agarose, annealing 69°C.

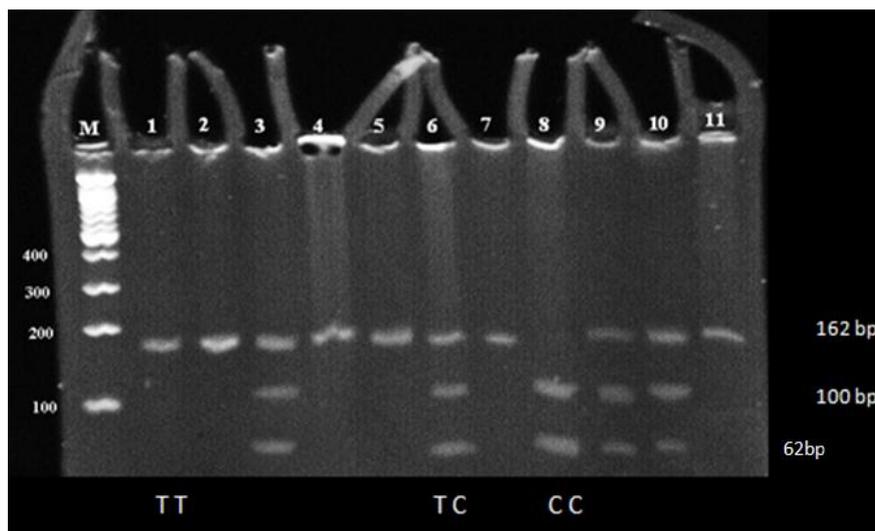


Figure (5): Electrophoresis pattern of PCR-RFLP for PCR product (162 bp) of *TSHR* gene polymorphism. Lane (1-4): control group. Lane (5-11): hypothyroidism patients, Lane (3, 6, 9, 10): heterozygote (TC) genotype, Lane (1, 2, 4, 5, 7, 11): homozygote (TT) genotype, lane (8): homozygote (CC) genotype. Restricted fragments with restriction enzyme *ALU1* were electrophoresed in 8% polyacrylamide gel, 0.5X TBE, 150V, 20mA for 3 hours.
Samples of Analysis for Hardy-Weinberg equilibrium

The samples show there was no significant deviation from equilibrium of Hardy-Weinberg and this lead to assume that the samples represent the population and there is no significant error in the genotyping technique used in this study as shown in the table (2).

Table (2): Exact test for Hardy-Weinberg equilibrium.

	Genotypes			Alleles		P-value*
	T/T	T/C	C/C	T	C	
All subjects	73	20	1	166	22	1
Patients	34	16	1	84	18	1
Control	39	4	0	82	4	1

* The two-tailed P value of Fisher's exact test

Allele Frequency

The result shows that T allele frequency in Iraqi population is 95% and C allele frequency is 5% this frequency is compared to other population frequencies as shown in the figure (6).

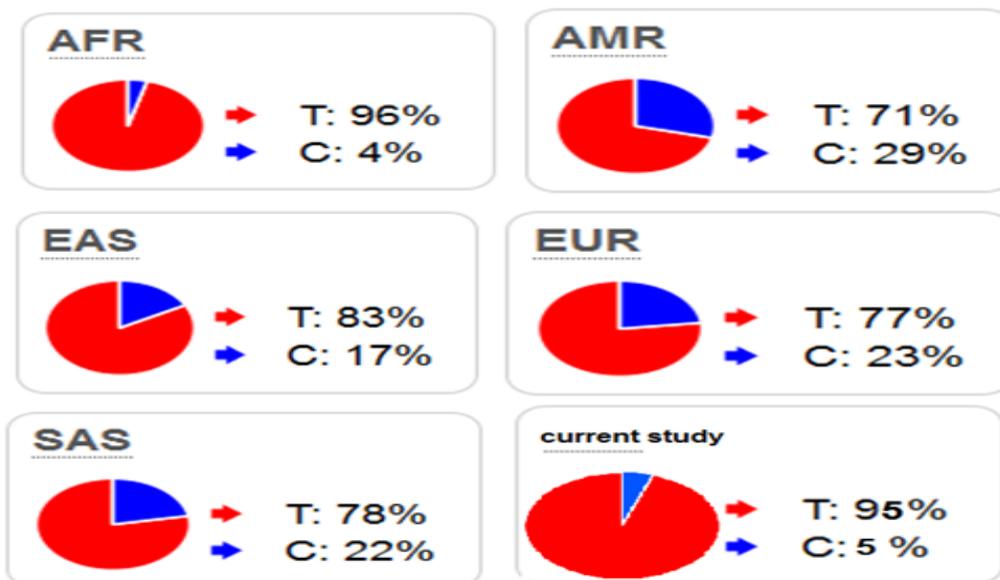


Figure (6): *TSHR* rs2268458 alleles frequencies in Babylon province population compared to several populations. The data and figures from other than studying population (current study) are adapted from 1000 Genomes Project Phase 3. (AFR= Africans; AMR= Americans; EAS= East Asians; EUR= Europeans; SAS=South Asians).

Allelic Association of *TSHR* gene rs2268458 in Hypothyroidism

The result of allelic association showed that the allele T was less frequent in hypothyroidism patients 0.82 when compared to control group 0.95 (OR= 0.228, CI=0.074-0.701) while the allele C was more frequent in hypothyroidism patients 0.18 when compared to control group 0.05 (OR=4.393, CI=1.42-13.53), by the other words allele C has a significant association with hypothyroidism disease which mean that the individual who carries C allele was susceptible to hypothyroidism by (OR = 4.393) and (CI=1.42-13.53) as shown in table (3).

Table (3): Genotype of *TSHR* gene rs2268458 polymorphism with the Allelic association.

Allele	Patients		Control		OR (95% CI)	P-value*
	Count	Proportion	Count	Proportion		
T	84	0.82	82	0.95	0.228 (0.074-0.701)	0.0057
C	18	0.18	4	0.05	4.393 (1.42-13.53)	

* Pearson's goodness-of-fit chi-square.

Genotypes Association of rs 2268458 with Hypothyroidism

Allele C shows its pathological effect on different inheritance patterns in dominant, codominant and overdominant but its largest effect is in the dominant model of inheritance (P=0.006, OR= 4.87, CI=1.49-15.90) and from this, it can be assumed that allele C is a pathological allele and inherited in a dominant model. The association of genotype under different models of inheritance are detected in the Table (4).

Table (4): Genotypes association of rs 2268458 with hypothyroidism under different patterns of inheritance

	Genotype	Patients	Control	OR (95% CI)	P-value*
Codominant	T/T	34 (66.7%)	39 (90.7%)	1.00	0.007
	T/C	16 (31.4%)	4 (9.3%)	4.59 (1.40-15.06)	
	C/C	1 (2%)	0 (0%)	3.435(0.135-87.094)	
Dominant	T/T	34 (66.7%)	39 (90.7%)	1.00	0.006
	T/C-C/C	17 (33.3%)	4 (9.3%)	4.87 (1.49-15.90)	
Recessive	T/T-T/C	50 (98%)	43 (100%)	1.00	1.00
	C/C	1 (2%)	0 (0%)	2.58(0.10- 65.08)	
Overdominant	T/T-C/C	35 (68.6%)	39 (90.7%)	1.00	0.011
	T/C	16 (31.4%)	4 (9.3%)	4.46 (1.36-14.61)	

The Concentration of Physio-Biochemical Parameters According to *TSHR* rs2268458 Genotype

The result of the study showed that there was no association between *TSHR* rs2268458 and thyroid function tests (TSH, fT₃, and fT₄) but there was a significant difference between the genotypes of this SNP and each of the following: BMI, CT, and CYP1A1, where TC and CC genotypes were associated with high BMI, while TC genotype was associated with high CT and CYP4501A1 levels, as shown in table (5).

Table (5): The concentration of physio-biochemical parameters according to *TSHR* rs2268458 genotype.

Variable (Mean±S.E)	Patients			LSD
	TT N=34	TC N=16	CC N=1	
BMI (Kg/m²)	29.16±5.27	32.98±4.26	38.28±0.0	2.965
TSH (μIu/ml)	14.37±4.87	14.38±4.92	5.51±0.0	14.816
fT₃ (Pg/ml)	1.58±0.89	1.58±0.88	1.13±0.0	0.528
fT₄ (ng/dL)	0.61±0.29	0.55±0.22	0.49±0.0	1.164
OPN (ng/ml)	3.39±1.66	4.25±1.55	5.76±0.0	3.967
CT (Pg/ml)	51.99±4.14	59.19±14.78	49.84±0.0	5.328
CYP (ng/ml)	7.42±0.84	7.91±1.02	5.03±0.0	0.336
Ca (mg/dL)	8.22±1.17	8.11±1.24	8.03±0.0	0.711
P (mg/dL)	4.39±2.35	3.95±0.78	4.89±0.0	1.187

CRD, P≤0.05

Correlation between rs2268458 genotypes of *TSHR* gene and Physio- Biochemical parameters

The results of the study show that in TT genotype there was a significant positive correlation between OPN and each of the following: fT₄ level (R²= 0.127, p=0.038) and CYP1A1 level (R²=0.187, p=0.01) as shown in figure (7).

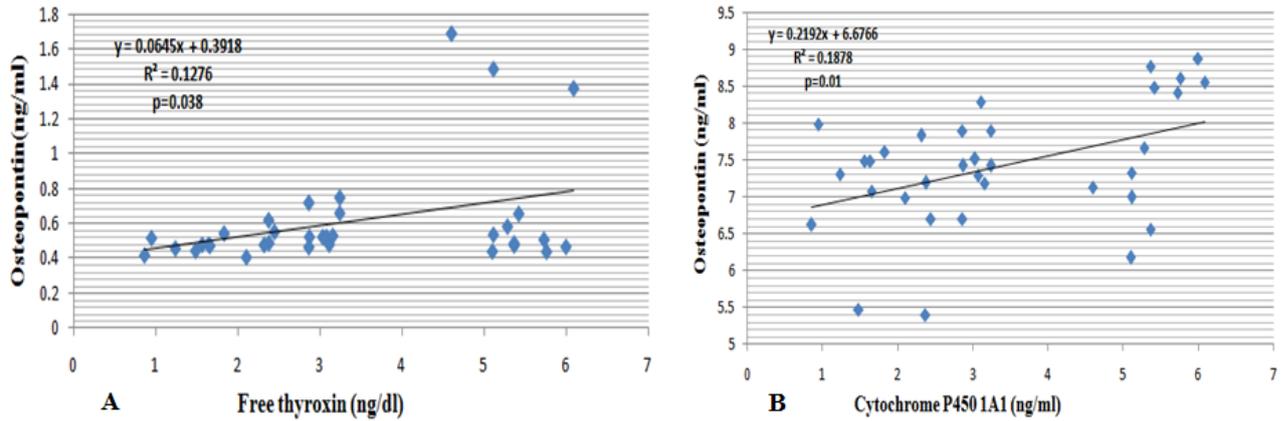


Figure (7): Linear regression analysis revealed positive correlation between osteopontin and free thyroxin (A) and cytochrome P450 1A1 (B) in TT genotype.

In addition, there was a significant positive correlation between OPN and CT mean levels in TC genotype ($R^2 = 0.251$, $p = 0.048$) as shown in the figure (8).

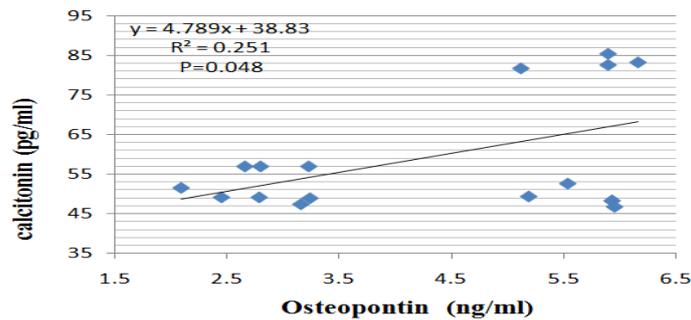


Figure (8): Linear regression analysis revealed a positive correlation between calcitonin and osteopontin in TC genotype.

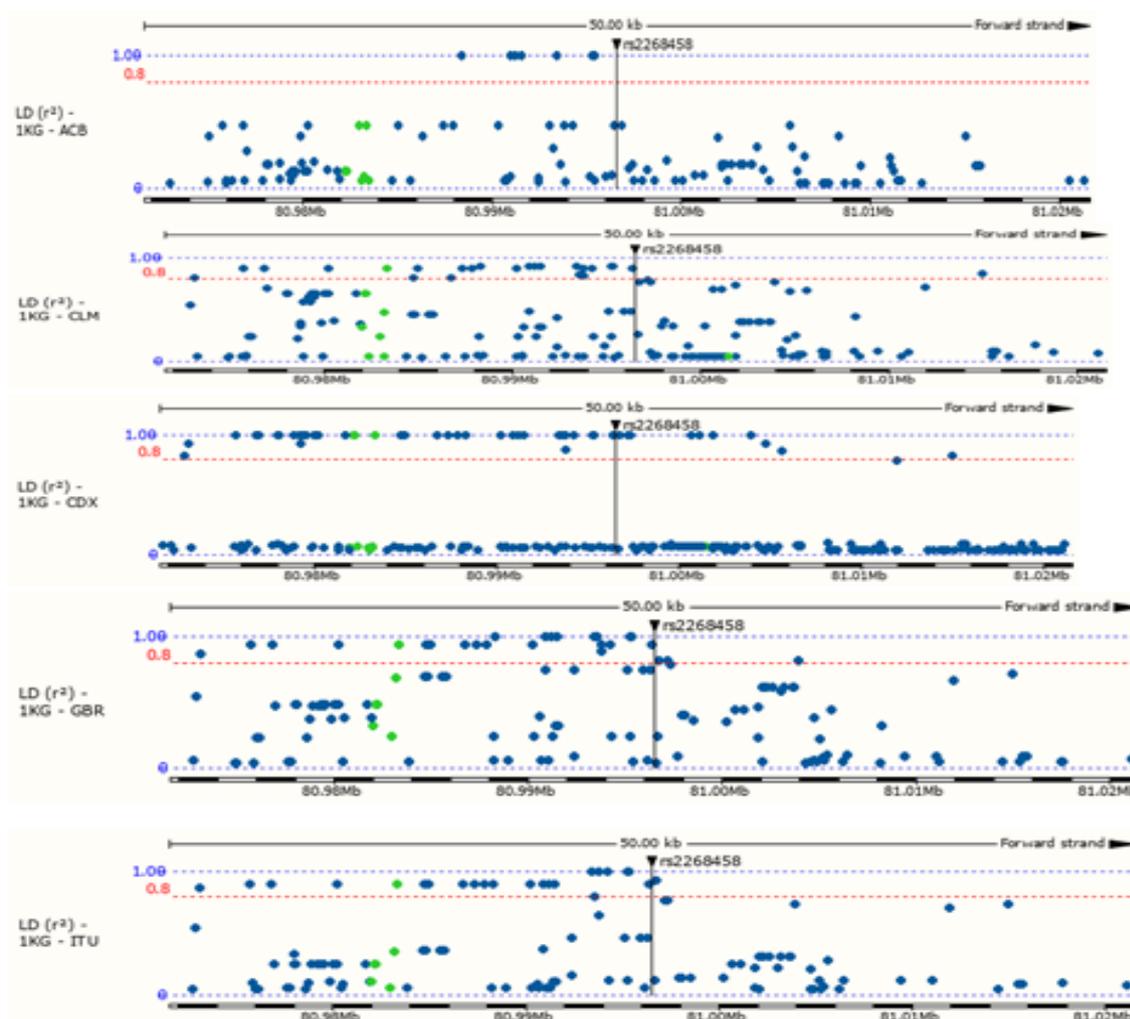


Figure (9): Manhattan plots of linkage disequilibrium (LD) in different population estimated by r^2 for variants linked to rs2268458 variant (ACB: African Caribbean in Barbados, CLM: Colombian in Medellin, Colombia, CDX: Chinese Dai in Xishuan-gbanna, GBR: British in England and Scotland, ITU: Indian Telugu in the UK) (Ensembl release 91 - December 2017 © [EMBL-EBI](#)).

Discussion

Genetics had an important role in both determinations of TSH and thyroid hormone concentrations and vulnerability to thyroid disease. Heritability studies have referred that up to 67% of TSH and thyroid hormone concentrations in circulation are genetically settled and TSH receptor gene is one of the candidate genes which contribute in the variability of thyroid hormone concentrations [9]. TSH regulates the secretion of thyroid gland and growth through binding with its receptors (TSHR) [6] which are found not only in thyroid follicular cells, besides finding in osteoclasts and osteoblasts and several studies regarded that TSH as a negative regulator of bone turnover [10], [11]. According to the previous study, genetic variants in the *TSHR* gene was described to be associated with the risk of thyroid diseases [7].

The *TSHR* rs2268458 was confirmed its association with Graves' Disease (GD) in

Caucasians [8]. The current study has been discussed whether or not the association of *TSHR* rs2268458 with thyroid function tests (FT3, FT4, and TSH) as well as other physio-biochemical parameters in Iraqi hypothyroidism women in Babylon province. Many studies have investigated the role of mutations and the polymorphism in *TSHR* gene in the development of different thyroid diseases [12], [13], [8], [14].

The result of this study show that allele C has a pathological effect on Iraqi hypothyroidism women and its largest effect in the dominant model, so TC and CC genotypes are associated with hypothyroidism disease and this

result disagrees with [15] who reported that *TSHR* rs2268458 polymorphisms were not associated with hypothyroidism disease in Lebanon population. This outcome indicates the supportive of genetic factors finding in this region that may be in linkage disequilibrium with the inheritance of different *TSHR* polymorphisms [16], so the difference between results can be explained by the differences in linkage disequilibrium in this polymorphism rs2268458 in different populations as shown in figure (9), where the figure demonstrates that this region of rs2268458 is highly polymorphic from population to population.

The result of this study showed that there was no association between rs2268458 polymorphism and thyroid function tests (TSH, fT3 and fT4) which may be due to that allele C may have Quantitative Trait Loci (QTL) which affect disease in quantization manner and need for QTL of another allele which may be different from population to another population as elucidated in figure (9) or may be due to low statistical power of our small sample size. These results agree with [17] who found a significant difference in the allelic frequency of the P52T polymorphism and did not discover any association between genotypes and serum TSH levels. Polymorphism does not essentially have to play a functional role [18]. In *TSHR* gene, many polymorphisms have been described and associated with a slight increase in TSH level [19] while other polymorphisms and mutations in other genes, such as PAX-8, FOXE1, and thyroid transcription factors TITF-1 could inhibit the expression of *TSHR* [20].

The result of the current study revealed that TC and CC genotypes were associated with high BMI, which may be due to allele C which has a significant association with hypothyroidism in this study and may act to disrupt the balance between energy expenditure and energy intake that result in obesity. In this study TC genotype was associated with high CT and CYP4501A1 levels which may be due to the changes in thyroid function in hypothyroidism patients can affect the osteomineral metabolism causing changes in the dynamics of calcitonin secretion [21] and there was another explanation that in this study allele C which has a significant association with hypothyroidism may be effective on the target genes for calcitonin and CYP4501A1 expression. In the previous study, total thyroidectomy could cause a significant increase in BMI and a significant decrease in thyroid hormones, OPN and calcium levels in Iraqi hypothyroid women [22].

The correlation study in TT genotype record a significant positive correlation between OPN and each fT4 and CYP4501A1 levels and this result agreed with [23] who found a positive correlation between osteopontin and thyroxin hormone and several studies reported that OPN was decreased in hypothyroidism [23], [24] which may be resulting from some processes of cell going on in the thyroid gland which probably decrease OPN receptor coexpression [23]. The significant positive correlation between OPN and CYP450 1A1 levels in this study accordance with [23], [25] who reported that CYP450 and OPN were decreased in hypothyroidism patients and this decreasing in CYP450 level may be because the low level of thyroid hormone in hypothyroidism patients which is responsible for regulation of expression of CYP through its control on levels of cellular CYP reductase, which had the ability to inactivate CYPs [26].

To our knowledge, this is the first study showing an association of *TSHR* gene rs2268458 polymorphism with hypothyroidism in Iraqi women. Our results suggest that the individual who carries C allele was susceptible to hypothyroidism by OR=4.393 CI=1.42-13.53. Also, TC and CC genotypes were associated with high BMI while TC genotype was associated with high calcitonin and CYP1A1 levels.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الخلاصة:

الهدف من الدراسة: لتقييم العلاقة بين تعدد الاشكال في الجين *TSHR* rs2268458 وبعض المعايير الفسلجية البايوكيميائية في النساء اللواتي يعانن من نقص الافراز الدرقي.

طرق العمل: تضمنت هذه الدراسة ٥١ امرأة تعاني من نقص الافراز الدرقي و٤٣ امرأة صحيحة كمجموعة سيطرة. تضمنت الفحوصات الفسلجية والبايوكيميائية كل من مؤشر كتلة الجسم، السايتركروم، الكالسيوم والفسفور. شملت الفحوصات الهرمونية كل من الاوستيوبونتين، الهرمون المحفز للدرقية والهرمون الدرقي الحر وهرمون الكالسيونين. تم اجراء التتميط للجين *TSHR* rs2268458 باستخدام تقنية ال PCR-RFLP.

النتائج: يرتبط تعدد الاشكال بال *TSHR* rs2268458 مع مرض نقص الافراز الدرقي في النساء العراقيات وبعد الاليل C عامل خطر لمرض نقص الافراز الدرقي. $OR= 4.393$, $CI= 1.42- 13.53$. حيث كانت الطرز الوراثة TC و CC مرتبطة بارتفاع مؤشر كتلة الجسم بينما ارتبط الطراز الوراثة TC مع المستويات العالية من السايتركروم *CYP4501A1* والكالسيونين.

الاستنتاج: ان تعدد الاشكال بالجين *TSHR* rs2268458 له علاقة بمرض نقص الافراز الدرقي في النساء العراقيات وان الاليل C يمكن ان يكون عامل خطر لبعض الاختلالات الهرمونية والمعايير الكيميائية الفسلجية في مريضات نقص الافراز الدرقي.

الكلمات الدالة: جين *TSHR*، rs2268458، نقص الافراز الدرقي، العراق.