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ORIGINAL ARTICLE

The Polymorphism Study of LEP and LEPR Genes in Association of its Serum Level in Iraqi Patients with Type 2 Diabetic

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ABSTRACT

Background: Diabetes is a major cause of mortality worldwide. There are several types of diabetes, with type 2 diabetes mellitus (T2DM) being the most common. Many factors, including environmental and genetic factors, are involved in the etiology of the disease. Numerous studies have reported the role of genetic polymorphisms in the initiation and development of T2DM. Aim of study to investigate the prevalence of single nucleotide polymorphisms in LEP gene (LEP 3′UTR A/C, −2548 G/A) and LEPR (K109R and Q223R) and their association with Leptin level and obesity

Methods: Here we recruited 150 patients with T2DM and 150 normal individuals. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism, BMI was calculated, and Leptin level was measured by ELISA. The polymorphism study was performed At Leptin -2548G/A and Leptin receptor LEPR Q223R. Statistical analyses were performed by spss19.0.

Results: Our data showed that there was a significant difference between BMI, HbA1c, Cholesterol, TG, HDL, LDL and Leptin level in T2DM and normal group, however there was no significant difference in gender of all individuals in two groups of T2DM and normal groups. The G-allele frequency was found to be significantly different between T2DM and control groups. In addition, the genotypic and allele frequencies of Leptin receptor LEPR Q223 Polymorphism in the study individuals have been presenteted. The Q-allele frequency was found to have no significant difference between T2DM and control groups. Our data clearly indicated that HbA1c significantly related with BMI. Similarly cholesterol level significantly related to BMI and HbA1c. There was a similar pattern between other parameters. However there were no significant relationship between HDL and other lipid profile. Moreover, the concentration of Leptin was 5.74± 1.27, 5.67± 1.24 And 4.09 ± 1.44 For genotypes of QQ, QR and RR, respectively. However there was no significant difference in normal group.

Conclusion: We found a significant association between the LEPR gene polymorphism and increased T2DM risk in the Iraqi population. Iraqi carriers of the G allele of LEPR gene polymorphism may be more susceptible to T2DM.

Keywords: Leptin, Leptin Receptor, Polymorphism, Type 2 Diabetes

INTRODUCTION

The diabetes classification includes a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion or insulin action. Such a defect increases the risk of cardiovascular disease, kidney failure, blindness, neuropathy and peripheral circulatory disease. It is estimated that diabetic patients will increase to 592 million by 2035 [1]. Type 2 diabetes mellitus (T2DM) accounts for more than 90% of diabetes cases and arises from complex interactions between environmental and genetic factors [2]. One such attractive and popular Single Nucleotide Polymorphism (SNP) candidate for obesity is the gene coding for leptin receptor. The leptin receptor gene polymorphism plays an important role in obesity and type 2 diabetes. But the role of this polymorphism is not yet studied in Iraqi population[3]. Hence, the study focused to explore the association of leptin and its receptor polymorphisms and its effects on leptin level in type 2 diabetes in both diabetic and non-diabetic subjects recruited from the local population of Iraq. Obesity is a common condition in industrialized societies and is increasing rapidly. Its etiology is complex and results from combined effects of genes, environment, lifestyle, and their interactions. Obesity has become a major issue because of its links to type 2 diabetes, hypertension, dyslipidemia, and insulin resistance syndrome[4]. The strength of the link between obesity and specific conditions varies. One of the strongest is the link with type 2 diabetes, which is primarily characterized by insulin resistance. Excess weight is the reason behind 64% of cases of diabetes in men and 77% in women[5]..About 118 candidate genes are so far associated with obesity. Some of the important candidate genes involved in causing obesity are the genes encoding leptin (LEP), leptin receptor (LEPR), melano cortin 4 receptor (MC4R), adiponectin (ADIPOQ), corticotrophin releasing hormone1 (CRHR1), prohormone convertase1 (PC1), pro-opiomelanocortin (POMC), and resistin (RETN)[6]. Among them, leptin and its receptor play the central role. Leptin, encoded by the obesity (LEP) gene, is expressed mainly in adipocytes. Their levels are highly dependent on presence of fats in the cell. It is shown to regulate satiety, energy expenditure, neuroendocrine function, and reproductive competence. The biologic activities of leptin on target tissues are carried out through selective binding to a specific receptor, LEPR. LEPR maps in humans to the 1p31 chromosome and has at least five isoforms. The structure of the leptin receptor is similar to that of the helical cytokine receptor (class I). Leptin receptors form homodimers, which are capable of activating Janus kinases. The Janus kinase is then able to start activators of transcription. Leptin signaling via the Janus kinases and activation of transcription system is largely associated with the long form (LEPR1) of leptin receptor [7]. Studies performed on mice showed that the LEPR1 is important for transmitting the leptin signal to the cells and is located predominantly in the hypothalamus and not in other tissues. However, the short forms are expressed throughout the body, especially in the kidney, lungs, and choroid plexus. Several polymorphisms are commonly occurring in LEPR gene, which cause either synonymous or non synonymous substitutions. The role of homozygosity for inactivating mutations of the leptin receptor (LEPR) in producing extreme obesity syndromes in laboratory animals is established[8]. Additionally, a small number of extremely obese humans from consanguineous pedigrees have been identified, who are obese due to homozygosity for inactivating mutations of LEPR. Heterozygosity for LEPR mutations in mice and rats also results in increase in fat stores[9,10]. The question of whether more common polymorphisms of the LEPR gene confer increased susceptibility to obesity and its associated morbid disorder type 2 diabetes remains open in Iraqi population. Leptin is an endocrine hormone and a member of the long-chain helical cytokine family. It has several effects such as regulating food intake, energy expenditure, body weight and immune responses. Leptin effects are mediated by its receptor, which is located in the central nervous system and other tissues, including adipocytes and endothelial cells[4]. To date many studies showed that Leptin and Leptin receptor are potentially related to the pathophysiology of Type 2 Diabetes. But there is no evidence to evaluate polymorphism of Leptin and Leptin

receptor in these patients. On the other hand there is no evidence to determine how these polymorphism affect serum level of Leptin. Here we aimed to study the relationship between two SNPs (single nucleotide polymorphisms) in Leptin (−2548 G/A (rs7799039) and 3′UTR A/C (rs11761556) and two SNPs in Leptin receptor rs1137101 (Gln223Arg) and rs1137100 (Arg109Lys) on Leptin level in Iraqi patients with Type 2 Diabetes.

MATERIALS AND METHODS

The patients with type 2 diabetes written consent started on July of 2020. 150 normal individuals were also enrolled in this study. Blood samples were taken and transferred to -20 ° C. Age, sex, and laboratory indices were recorded. The prepared blood samples were removed from -20 ° C and placed under sterile conditions on an ice container. After making sure that the samples melted, Serum leptin was quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (DRG) according to the manufacturer's instructions. All samples were analyzed in duplicate. Genomic DNA was isolated from blood leukocytes using the commercial DNA isolation kit. The G2548A polymorphism of the LEP gene was determined using PCR–RFLP method. LEP gene promoter was amplified using the following primer pairs (51) : forward, 5-TTT CCTGTAATTTTCCCGTGAG-3; and reverse, 5-AAA GCAAAGACAGGCATAAAAA-3. PCR was carried out in a final volume of 25 µL containing 100 ng genomic DNA, 1.5 mmol/L MgCl2, 0.5 mmol/L of each dNTPs, and 0.5 pmol of each primer. After an initial denaturation of 3 minutes at 95°C, the samples were subjected to 30 cycles at 95°C for 40 seconds, 56°C for 50 seconds, and 72°C for 50 seconds, with a final extension of 10 minutes at 72°C. The 242 bp product was digested with HhaI for 2 hours at 37°C. The products were 181 and 61 bp for wild type; 242, 181, and 61 bp for heterozygotes; and 242 bp for homozygotes. The Q223R polymorphism of the LEPR gene was evaluated using PCR-RFLP method. Exon 4 was enlarged 8 using the following primer couples (52):

forward, 5-GCC TAATCCAGTATTTTCATATCTG-3; and reverse, 5-GCCACTCTTAATACCCCCAGTAC-3. The 416 bp.

Statistical analysis: Statistical analysis was performed via spss22.0. The quantitative parameters was compared using Student's test and reported as mean ± SD, and if they was in Gaussian distribution, they were compared using Mann-Whitney U test and reported as median [min-max] if not. Categorical variables were analyzed using the chi-square test. We used snip Analyzer 2 program to check Hardy-Weinberg equilibrium for both genotype and haplotype frequencies. snip Analyzer 2.0 is a web-based integrated workbench for linkage disequilibrium analysis and association analysis. Correlation between LEP and other biological and anthropometric parameters were studied using Spearmen test. The odds ratios (ORs), two-tailed P-values and 95% confidence interval (CI) were calculated as a measure of the association of the SNPs and haplotype with the presence of diabetes and were adjusted to potential confounder parameters (P < .25) by binary logistic regression. A P-value of <.05 was considered statistically significant for all tests.

RESULT

This study included 300 individuals including 150 normal and 150 T2DM patients. All individuals were divided into 4 age groups. Pearson chi square test of independence were performed. As indicated in Table 1 there was a significant difference between age groups in T2DM and normal group (P= 0.001), however there was no significant difference in gender of all individuals in two groups of T2DM and normal group

As indicated in Table 2 there was a significant difference between BMI, HbA1c, Cholesterol, TG, HDL, LDL and Leptin level in T2DM and normal group (Independent t-test, P= 0.001), however there was no significant difference in gender of all individuals in two groups of T2DM and normal groups

Table 1: Demographic characters of individuals

Demographic characters		T2DM (N=150)		Normal (N=150)		P-value©
		N	%	N	%	
Age group	20-29	0	0	3	2.0	0.001**
	30-39	11	7.3	67	44.7	
	40-49	99	66.0	72	48.3	
	50-59	40	26.7	8	5.0	
Gender	Male	77	51.3	86	57.3	0.140 n.s
	Female	73	48.7	64	42.7	

©Pearson chi square test of independence was used, n.s: Not significant (P > 0.05), *** Highly significant (P < 0.001)

Table 2: Clinical characterizations of individuals

Demographic characters	Abnormal (N=150)			Normal (N=150)			P-value©
	Mean	±	SD	Mean	±	SD	
BMI	34.92	±	2.96	23.27	±	1.27	0.001**
HbA1c	9.82	±	1.48	4.87	±	0.51	0.001**
Cholesterol	275.8	±	23.45	160.7	±	11.13	0.001**
T.G	301.9	±	26.61	121.1	±	18.36	0.001**
HDL	28.09	±	1.11	35.75	±	3.40	0.001**
LDL	189.6	±	20.82	100.9	±	13.37	0.001**
VLDL	60.34	±	5.69	24.26	±	3.65	0.001**
Leptem	6.114	±	1.141	5.36	±	0.85	0.001**

©: Independent t-test were used to test between groups, n.s: Not significant (P > 0.05), *** Highly significant (P < 0.001)

Frequency of Alleles in control groups: The genotypic and allele frequencies of Leptin -2548G/A Polymorphism in the study patients have been shown in Table 3 and figure 1. The G-allele frequency was found to be significantly (0.001) different between T2DM and control groups (58% vs 34%, respectively). In addition, the genotypic and allele frequencies of Leptin receptor LEPR Q223R Polymorphism in the study individuals have been presented in Table.4 and figure 2. The Q-allele frequency was found to have no significant difference between T2DM and control groups (44% vs 54%, respectively).

Table 3: Frequency of alleles of Leptin and its genotypes in all individuals

-2548G/A allele	Normal control (n=150)	Diabetic (n=150)	P value
GG	51 (34%)	87 (58%)	0.001***
GA	78 (52%)	51 (34%)	0.001***
AA	21 (14%)	12 (8%)	0.095 n.s
G allele	180 (60%)	225 (74.5%)	0.012*
A allele	120 (40%)	75 (25.5%)	0.012*

¥: Z-test of two proportions were used, *: significant (P < 0.05), *** Highly significant (P < 0.001)

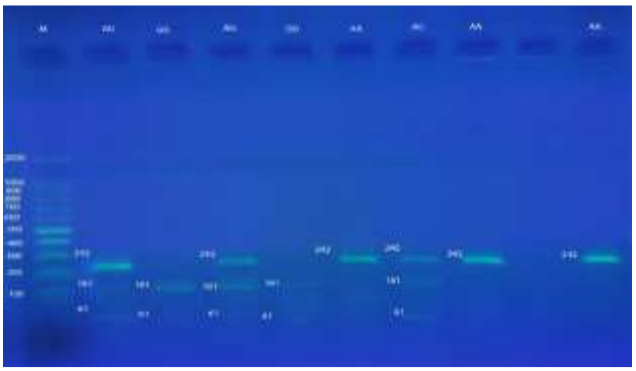

Figure 1: gel electrophoresis shows band pattern for leptin gene G-2548A/alleles.

Table 4: Frequency of alleles of LEPRQ223R and its genotypes in all individuals

LEPR Q223R alleles	Diabetic (n=150)	Normal control (n=150)	P value
QQ	66 (44%)	81 (54%)	0.246 n.s
QR	54 (36%)	54 (36%)	1.00 n.s
RR	30 (20%)	15 (10%)	0.086 n.s
Q allele	186 (62.0%)	216 (72%)	0.077 n.s
R allele	114 (38.0%)	84 (28%)	1.00 n.s

proportion z-test allows you to compare two proportions to see if they are the same. The null hypothesis (H0) for the test is that the proportions are the same

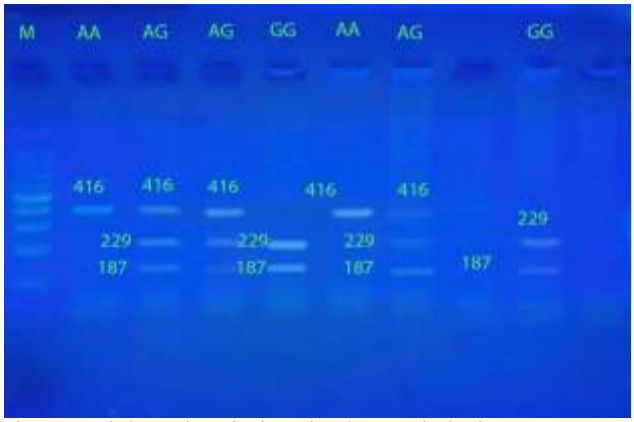

Figure 2: Gel electrophoresis shows band pattern for leptin receptor Q-223R (G>A) alleles, and molecular weights

Relationship between parameters in all patients: As indicated in Figure 3 the regression analysis between all parameters were performed. Our data suggested that HbA1c significantly related with BMI (P=0.001). Similarly, cholesterol level significantly related to BMI and HbA1c. There was a similar pattern between other parameters. However, there were no significant relationship between HDL and other lipid profile.

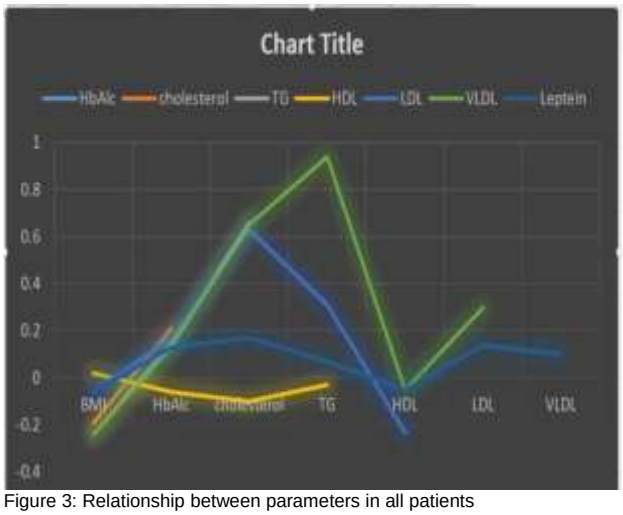

Figure 3: Relationship between parameters in all patients

Leptin concentration in different Leptin genotypes: AS presented in Fig 4 there was a significant relationship between Leptin concentration and various Leptin polymorphism in normal group. The concentration of Leptin was 2.81± 1.04, 2.85± 0.82 And 1.52 ± 0.73 For genotypes of GG, GA and AA, respectively. However, there was no significant difference in patients with T2DM

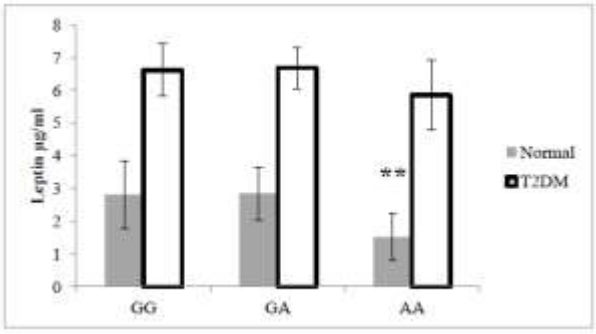

Figure 4: Leptin concentration in different Leptin genotypes, Leptin concentration was assessed in different polymorphism of Leptin in normal individuals and patients with T2DM.

Leptin concentration in different Leptin receptor genotypes: AS presented in Fig 5 there was a significant relationship between Leptin concentration in different Leptin receptor polymorphism in patients with T2DM. The concentration of Leptin was 5.74± 1.27, 5.67± 1.24 and 4.09 ± 1.44 For genotypes of QQ, QR and RR, respectively. However, there was no significant difference in normal group.

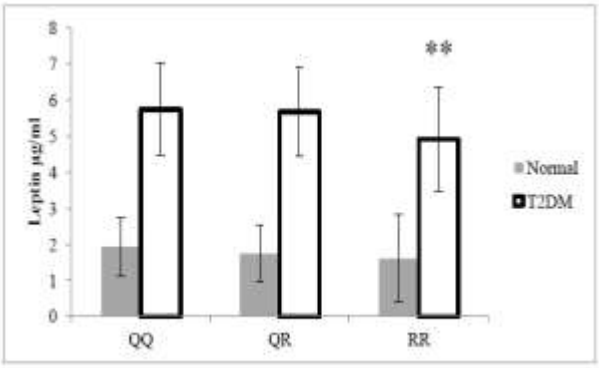

Figure 5: Leptin concentration in different Leptin receptor genotypes, Leptin concentration was assessed in various polymorphism of Leptin receptor in normal individuals and patients with T2DM.

Leptin concentration in different Leptin receptor genotypes: As described in Table 4 our fining showed that there was no significant difference between combination of both genotypes in normal individual however 44% of patients have QQ/GG genotypes (P=0.001, Table 5.

Table 5: Combination of Leptin and Leptin receptor in normal group

	QQ		QR		RR		Total	
	N	(%)	N	(%)	N	(%)	N	(%)
GG	27	(18.0%)	21	(14.0%)	3	(2.0%)	51	(34.0%)
GA	39	(26.0%)	30	(20.0%)	9	(6.0%)	78	(52.0%)
AA	15	(10.0%)	3	(3.0%)	3	(2.0%)	21	(14.0%)
Total	81	(54.0%)	54	(36.0%)	15	(10.0%)	150	(100)
Chi-Square Tests			12.772		P-value	0.173 N.S		

Table 6: Combination of Leptin and Leptin receptor in patients with T2DM

	QQ	QR	RR	Total
	N (%)	N (%)	N (%)	N (%)
GG	66 (44.0%)	17 (11.3%)	4 (2.7%)	87 (58.0%)
GA	5 (3.3%)	27 (18.0)	19 (12.7%)	51 (34.0)
AA	1 (0.7%)	7 (4.7)	4 (2.7%)	12 (8.0%)
Total	72 (72%)	51 (34.0)	27 (18.0%)	150 (100%)
Chi-Square Tests		199.9	P-value0.001 **	

DISCUSSION

T2DM is a polygenic disease which is closely related with obesity, hypertension, gout, and lipid metabolism disorder. T2DM is clinically called insulin resistance syndrome, the core of which is the IR. Recently many studies have shown that the leptin resistance might still exist in the IR patients at the same time. Leptin is a fat-derived hormone which could play a role in the regulating the energy metabolism and body lipid homeostasis after it is combined with the LEPR located in the hypothalamus and adipose tissue. Hence, the LEPR gene is also called T2DM gene(1). Our data showed that there was a significant difference between age groups in T2DM and normal group, however there was no significant difference in gender of all individuals in tow group of T2DM and normal group. In addition there was significant difference between BMI,HbA1c,Cholestol,TG.HDL,LDL,and leptin inT2DM and normal group. howere there was no significant difference in gender of all individuals in two group of T2DM and normal group the genotypic and allele frequencies of leptin -2548G/A polymorphism in the study patients have been shown in Table 3.The G-allele frequency was found to be significantly different between T2DM and control group .In addition ,the genotypic and allele frequencies of leptin receptor LEPR Q223 polymorphism in the study individuals have been presenteted .The Q-allele frequency was found to have no significant difference between T2DM and control groups .Our data clearly indicated that HbA1c significantly related with BMI .Similarly cholesterol level significantly related to BMI and HbA1c .There was a similar pattern between other parameters .However there were no significant relationship between HDL and other lipid profile. Morever, the concentration of Leptin was 5.78+_ 1.27, 5,67+_ 1.24 And 4.09+_ 1.44 for genotypes of QQ, QR and RR, respectively. However there was no significant difference in normal group.

What mechanisms can explain the increased risk of T2DM associated with this polymorphism? One possible mechanism is that the glutamine residue may cause reduced LEPR expression on the plasma membrane impairing signal transduction through the receptor. It also could increase risk of T2DM by influencing neurological function of the vagus nerve. The vagus nerve sensibility for the insulin secretion were reduced and further led to the IR, disrupting, not only glucose metabolism, but also fat metabolism. The IR not only makes the glucose metabolism disturbance, but also the fat metabolism disturbance. Although these mechanisms have yet to be demonstrated physiological, they provide a plausible rationale for the results from this study(2).

Liu et al and Su et al. both performed meta-analysis on the association between LEPR Gln223Arg gene polymorphism and T2DM in 2015 and 2016, respectively (3, 4). They both concluded that LEPR Gln223Arg gene polymorphism had no effect on the susceptibility with T2DM. Both of these papers, however, do not take into account differences in ethnicity. Liu's meta-analysis grouped all Asians, including Koreans, Indians, Malaysia, and Chinese in to a single subgroup while Su's meta-analysis analyzed data that combined Asians and Europeans. These meta-analyses also included studies with the controls' genotype number that deviated from HWE, such as individual studies by Zhang, Zhao, and Liu (5, 6).

These factors combined may help account for the differences in results and also lend further credibility to the results of this study.

CONCLUSION

we found a significant association between the LEPR gene polymorphism with increased T2DM risk in the Iraqi population. Iraqi carriers of the G allele of LEPR gene polymorphism may be more susceptible to T2DM.

Recommendation: We recommend that future study to use more sample size from all providence of Iraq. Because, Iraqi population may have different race in different provinces. In addition Leptin and Leptin Receptor polymorphism should be used to screen T2DM patients.

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