Genetic Variation in Leptin Receptor Gene in Diabetic and Non-Diabetic Obese Subjects from Erbil City

التغايرات الجينية لجين مستقبل هرمون الليبتين (*LEPR* Gln223Arg) في افراد مصابين وغير مصابين بالسكري والذين يعانون السمنة في اربيل

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Abstract

There have been numerous genetic causes of obesity specifically leptin, genetic variants of the leptin receptor gene (*LEPR* Gln223Arg) which appeared to be polymorphismized (A>G; rs1137101) have been implicated in the pathogenesis of obesity in several populations, although no association has been evidenced in other regions in the world. In this study, the association between *LEPR* Gln223Arg polymorphism with Body Mass Index (BMI) and plasma leptin levels in obese diabetic and obese non-diabetic adults who are randomly selected from Erbil city is evaluated. Blood samples were collected for DNA extraction, and plasma leptin measurements. *LEPR* (A > G; rs1137101) genotypes were identified by a PCR- RFLP. The results show that plasma leptin concentrations increased with body mass index, and in obese diabetic group was more than two-fold increases p=0.001 when compared to those of obese non-diabetic patients. *LEPR* (A > G; rs1137101) gene polymorphisms did not found associated with BMI in the whole studied population. Furthermore an increased frequency of the GG genotype in the female control group 32.1 compared to obese group 19.2, but the frequency did not significant (OR= 1.38: 95% CI; 0.74-2.03, P=0.07) which indicated that this genotype might be associated with a protective effect against obesity in female only and that this effect was independent of diabetes. Further analysis of a larger population is required to confirm the biological relevance of this polymorphism for obesity in the Kurdish population.

Keywords: Leptin, Gene polymorphism, Body mass index, Obesity, Diabetes milletus, PCR-RFLP.

الملخص

هناك أسباب وراثية عديدة تسبب السمنة ومنها اللبتين، المتغيرات الجينية لجين مستقبل هرمون الليبتين (*LEPR* Gln223Arg) المتعدد الاشكال (A >G; rs1137101) مرتبطة بالسمنة في العديد من السكان، على الرغم من عدم وجود هذه العلاقة في مناطق أخرى. في هذه الدراسة تم دراسة العلاقة بين تعدد الاشكال *LEPR* Gln223Arg مع مؤشر كتلة الجسم ومستويات اللبتين في البلازما لدى المجموعة التي تعاني من السمنة والمصابين بداء السكري مع أولئك غير المصابين بالسكري ويعانون من السمنة ومقارنتهم بالأصحاء من المواطنين الذين تم اختيارهم عشوائيا من مدينة أربيل. جمعت عينات الدم لاستخلاص الحمض النووي، والبلازما لقياس اللبتين، حدد التغاير الجيني (A > G; rs1137101) بوساطة تفاعل التكراري المتسلسل وتقنية الحصول على قطع الدنا متباينة الاطوال باستعمال انزيمات التقييد PCR- RFLP. أظهرت النتائج أن تركيزهرمون اللبتين البلازمي يزداد مع مؤشر كتلة الجسم، وكانت أعلى بمقدار ضعفين p=0.001 لدى المجموعة الذين يعانون من السمنة والمصابين بداء السكري بالمقارنة مع أولئك المرضى غير المصابين بالسكري ويعانون من السمنة المفرطة ،كما ان تعدد الاشكال لجين اللبتين (A >G; rs1137101) ليس له علاقة مع موشر كتلة الجسم في عينة الدراسة. زيادة التكرار الاليلي للنمط الوراثي GG في مجموعة السيطرة لعينات الاناث كانت 32.1 مقارنة مع مجموعة الذين يعانون السمنة والبالغة 19.2 ولكن التاثير لم يكن معنويا (OR= 1.38: 95% CI; 0.74-2.03, *P*=0.07) مما يدل ان النمط الوراثي ذو تاثير وقائي ضد فرط السمنة في الاناث فقط وان هذا التاثير مستقل عن مرض السكري. لدراسات مستقبلية هناك حاجة لتحليلات اضافية لمجموعة اكبر من السكان لتاكيد الاهمية البايوجية لتعدد الاشكال لفرط السمنة في سكان الاكراد .

الكلمات الدالة: اللبتين، المتغيرات الجينية، مؤشر كتلة الجسم، السمنة، داء السكري، تفاعل التكراري المتسلسل PCR- RFLP

Introduction

Obesity and diabetes mellitus are essential public health concerns throughout the world, because of their increasing occurrence and prevalence. Obesity is the most important risk factor for type 2 diabetes mellitus and most patients with diabetes are overweight or obese. It is well known that excess bodyweight induces insulin resistance, which is a characteristic feature of type 2 diabetes. Obese individuals have an increased risk of morbidity because of the various related disorders, including diabetes, cardiovascular events, stroke, cancer, and obstructive sleep apnea [1,2]. Leptin is a metabolic and neuroendocrine hormone mainly produced by adipocytes [3], but it is also detected in several tissues such as lymphoid tissues, placenta, and ovaries [4].

Leptin is an important regulator of adipose tissue mass and of body weight; it works by inhibiting food intake and stimulating energy expenditure. Since the discovery of leptin, it has been expected the therapeutic potential for obesity and diabetes. Leptin is known as a key appetite-regulating hormone, which effects on appetite, energy expenditure, and glucose metabolism [5]. Leptin receptors are present in the β-cell, it considered as an important regulator of pancreatic β-cell function at different levels including insulin secretion, insulin gene expression, cell growth, and apoptosis. This hormone also has a key function in the regulation of glucose homeostasis due to different levels of modulation of the pancreatic β-cell, it has been proposed that alterations in leptin signaling in the β-cell might be involved in diabetes in obese individuals [6,7]. Some polymorphic genes involved in the regulation of leptin have been investigated as possible factors associated with obesity [8]. leptin receptor gene Gln223Arg polymorphism (*LEPR* Gln223Arg) has been mentioned as one of the factors of genetic predisposition to overweight [9,10]. Since the *LEPR* Gln223Arg has a functional importance for obesity, it could play a significant role in type 2 diabetes mellitus and pathophysiology of human obesity [11]. Furthermore, they may share a common genetic background; that is, the risk alleles for obesity may also be involved in the increased risk of developing type 2 diabetes [12]. In the Kurdistan regional of Iraq, few epidemiologic studies have been done to assess the prevalence of obesity and type II diabetes with the leptin receptor, it will be important to clarify the functionality of different genetic variants, since several studies have found significant associations linking them to several traits of obesity, diabetes or the metabolic syndrome [10]. For our knowledge, no data are yet available in Erbil/ Kurdistan regional of Iraq on the association of the *LEPR* Gln223Arg genotypes with obesity and diabetes. Therefore, the present study investigated the association between *LEPR* Gln 223 Arg (A > G; rs1137101) polymorphism in obese diabetic and obese non-diabetic subjects from Erbil city, and realize the possibility that the risk alleles for obesity involve in the increased risk of developing type 2 diabetes.

Materials and Methods

Study participants

The study group composed of 150 Kurdish volunteers from Erbil city, 50 obese diabetic (29 men and 21 women) the mean age is 35.02±7.03, 50 obese non-diabetic (24 men and 26 women) the mean age is 29.16±25, the other 50 subjects (22 men and 28 women) are considered as the control group (non-obese and non-diabetic) with the mean age 27.21±2.59. Body mass index (BMI) is taken from each participant and calculated as weight (kg) divided by height (m) squared. Subjects in this study were classified as normal weight (control group / non-obese group) (BMI< 25kg/m2), and obese group (BMI> 25kg/m^2). Pregnant females were excluded from the study.

Blood samples collection and biochemical data

Fasting (12 hours) venous blood samples were obtained in the early morning into tubes that contained EDTA-Na_2 as an anticoagulant. Plasma was separated and used for leptin determination, and cells were kept frozen at −20◦C for DNA extraction. Plasma leptin level (PLL) was determined using commercially available enzyme-linked immunoabsorbent assay (ELISA) kit (EIA-2395, DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions.

DNA extraction and polymorphism analysis

Genomic DNA was prepared from leukocytes using genomic DNA Mini kit (Gene aid Biotech Ltd), DNA purity and quantity were assessed by NanoDrop1000 spectrophotometer V 3.7 and checked by 0.7% Agarose gel electrophoresis. Leptin receptor gene polymorphism LEPR Gln223Arg (A > G; rs1137101) was assessed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Polymerase chain reaction for LEPR gene was performed on 50 ng of genomic DNA, as template. Other reagents were used, including 0.4 μM of each primer (F: 5`-CCTGCTTTAAAAGCCTATCC -3`) and (R: 5`-GCCACTCTTAATACCCCCAG-3`) and PCR (BioneerAccuPower® PCR PreMix). DNA amplification was performed with palm-cycler™ (Corbett research) DNA thermal cycler with 5 min of initial denaturation at 95°C followed by 35 cycles with denaturation for 1 min at 94°C, annealing for I min at 55°C, extension for 1 min at 72°C, and final extension for 10 min at 72°C. The 368 bp PCR product is digested with M*spI* restriction endonuclease (Promega, USA), that recognizes the restriction site (C/CGG). For this Single Nucleotide Polymorphism (SNP), the A allele lacks M*spI* restriction site. Thus, individuals carrying A allele show only one PCR product (368 bp), while those who carry G allele show two bands (245 and 123 bp) and individuals carring A and G alleles show three bands (368,

245 and 123 bp). Positive control for digestion reaction was used and 10 μL amplified DNA is digested with 1.0U of M*spI* for 4h at 37°C.

Detection of *LEPR Gln 223 Arg* polymorphism by electrophoresis

5 µl of the amplified PCR product (2% agarose gels) and restriction digestion products (3% agarose gels) were evaluated on ethidium bromide stained agarose gels, visualized and photographed.

Statistical analysis

Data analysis was carried out using SPSS version 11.5 (SPSS, Chicago, USA). Demographic details were compared using t-test for continuous data. Allele frequencies and genotype distribution of the population were compared by Chi square and/or fisher`s exact test. Odds ratios were calculated with a 95% confidence interval. In all cases statistical significance was determined at the P value < 0.05.

Results and Discussion

Anthropometric and biochemical data of obese non-diabetic group are shown in Table (1); there are significant differences between the obese group and control group in body mass index (BMI) and leptin P< 0.05. These results illustrate that plasma leptin concentration in obese non-diabetic group increase with body mass index.

Table (1): Anthropometric and biochemical data of the obese non-diabetic subjects

Variable	Control n=50	Obese Non-diabetic n=50	P value
Sex (M/F)	22/28	24/26	Ns
Age (years ±SD) :n	27.21±2.59	29.16±2.5	Ns
BMI	23.11±1.34	32.29±1.51	0.009
Leptin (ng/ml)	6.01±5.4	12.16±1.20	0.001

Table (2) shows anthropometric and biochemical data of obese diabetic subjects, the obese individuals' revealed mean age, BMI, and plasma leptin values higher than the non-obese group $p < 0.05$. Plasma leptin levels in obese diabetics (30.08±11.2) when compared to those of obese non-diabetic patients (12.16±1.20) show more than two-fold increases p=0.001, Table (1).

Table (2): Anthropometric and biochemical data of the obese diabetic subjects

Variable	Control n=50	Obese diabetic n=50	P value
Sex (M/F)	22/28	29/21	Ns
Age (years ±SD) :n	27.21±2.59	35.02±7.03	0.02
BMI	23.11±1.34	34.18±6.24	0.007
Leptin (ng/ml)	6.01±5.4	30.08±11.2	0.001

The genotyping results of leptin receptor rs1137101 variants are shown in Figure (1), the presence of A allele confirmed by the existence of a single band of 368 bp while double bands of 245 and 123 bp shows the presence of G allele. There are 2 bands for the G allele because this product contains a digestion site for the *MspI* enzyme, which is absent when A allele is present. Therefore, the PCR product containing the G allele is cleaved by *MspI* and produces 2 bands of low molecular weight. A single band of 368 bp shows the presence of A allele.

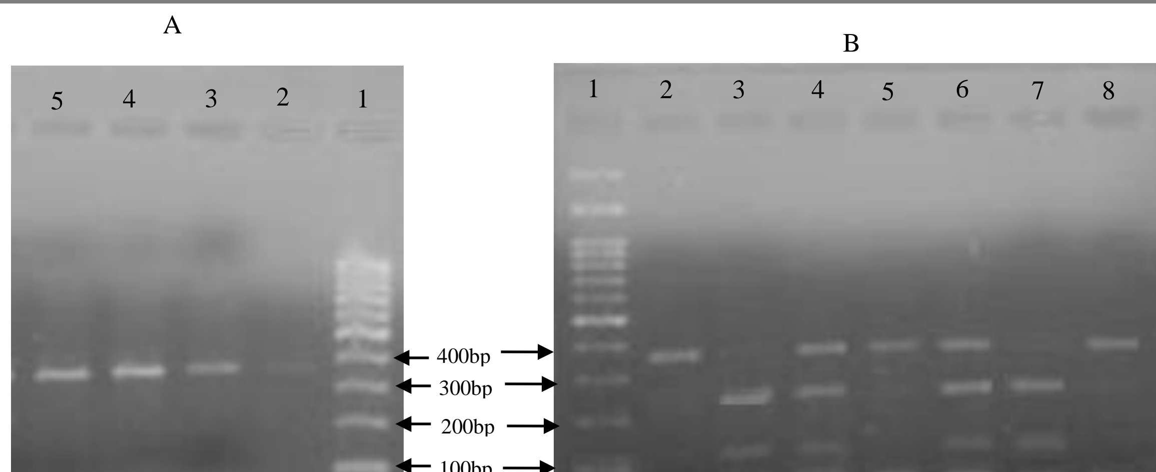

Fig. (1): A: Ethidium bromide stained agarose gel 2% (w/v) image showing PCR products for LEPR gene. Lane1:100bp DNA marker; lanes 2-5 (368 bp) LEPR gene.
B: Ethidium bromide stained agarose gel 3% (w/v) image showing RFLP products for LEPR gene. Lane1:100bp DNA marker; lanes 2,5,8 (368 bp) Homozygous AA genotype. Lanes 4,6: Heterozygous AG genotype produce three bands of size 368, 245 and 123 bp. Lanes 3, 7: Homozygous GG genotype produce two bands of size 245 and 123 bp

Table (3) shows genotype distribution and allele frequencies in obese non-diabetic male. The obese group genotyping are 50 % (n=12) homozygous AA, 37.5 % (n= 9) heterozygous AG and 12.5 % (n= 3) homozygous GG compared to the non-obese group (control) (45.5 % (n= 10) homozygous AA, 36.4 % (n=8) heterozygous AG, and the GG homozygous genotype is 18.1% (n=4). In control group, the frequency of the A and G alleles are 63.6 and 36.4 respectively compared to obese group (A and G alleles frequency are 68.8 and 31.2 respectively). In contrast, there are no significant differences in alleles frequencies between obese and non-obese group *P*=0.09.

Table (3): Genotypes and Allele frequencies in obese non-diabetic male

Genotypes	Frequencies % Control =22	obese non-diabetec =24	*P* Value	OR (95% CI)	RR (95% CI)
AA	10 (45.5 %)	12 (50 %)			
AG	8 (36.4 %)	9 (37.5 %)	^{a}0.5	1.3 (0.28-6.35)	1.22 (0.35-4.06)
GG	4 (18.1 %)	3 (12.5 %)	^{b}0.4	1.3 (0.32-6.2)	1.18 (0.39-3.4)
Alleles					
A	28 (63.6 %)	33 (68.8 %)	0.09	1.2 (0.42-2.9)	1.06 (0.4-1.96)
G	16 (36.4 %)	15 (31.2 %)			

OR: Odds Ratio. RR: Risk Ratio. CI: Confidence Intervals. ^{a}:AA *vs*. GG+AG, *P*-value Fisher`s Exact test. ^{b}: GG *vs*. AG+AA, *P*-value Person Chi-Square test.

When comparing the obese non-diabetic female and non-obese groups, the frequency of the homozygous AA, heterozygous AG, and the homozygous GG were 46.2 % (n=12), 34.6 % (n=9), and 19.2% (n=5) respectively, compared to control group are 35.8% (n=10), 32.1%, (n=9) and 32.1 % (n=9). There was an increased frequency of the GG genotype in the control group (32.1) compared to obese group (19.2), but the frequency did not significant (OR= 1.38: 95% CI; 0.74-2.03, P=0.07).

Table (4): Genotypes and Allele frequencies in obese non-diabetic female

Genotypes	Frequencies % Control =28	obese non-diabetec =26	*P* Value	OR (95% CI)	RR (95% CI)
AA	10 (35.8 %)	12 (46.2 %)			
AG	9 (32.1 %)	9 (34.6 %)	^{a} 0.7	1.3 (0.14-9.04)	1.1 (0.18-6.03)
GG	9 (32.1 %)	5 (19.2 %)	^{b} 0.07	1.38 (0.74-2.03)	1.44(0.9-3.8)
Alleles					
A	31 (55.4 %)	33 (63.5 %)	0.4	0.91 (0.35-2.26)	0.8 (0.39-1.73)
G	25 (44.6 %)	19 (36.5 %)			

OR: Odds Ratio. RR: Risk Ratio. CI: Confidence Intervals. ^{a}:AA *vs*. GG+AG, *P*-value Fisher`s Exact test. ^{b}: GG *vs*. AG+AA, *P*-value Person Chi-Square test.

Table (5) represents the comparison between obese diabetic male and control group. There were no significant differences in genotype distribution and allele frequency between study group and control.

Table (5): Genotypes and Allele frequencies in obese diabetic male

Genotypes	Frequencies % Control =22	Frequencies % obese diabetic=29	*P* Value	OR (95% CI)	RR (95% CI)
AA	10(45.5 %)	12 (41.4 %)			
AG	7(31.8 %)	9 (31 %)	a 0.09	0.66(0.16-3.04)	0.78(0.34-1.62)
GG	5 (22.7 %)	8 (27.6 %)	b 0.2	1(0.21-4.2)	1(0.52-1.9)
Alleles					
A	27 (61.4 %)	33 (56.9 %)	0.4	1.87(0.76-3.23)	1.07(0.76-1.21)
G	17 (38.6 %)	25 (43.1 %)			

OR: Odds Ratio. RR: Risk Ratio. CI: Confidence Intervals. a:AA *vs*. GG+AG, *P*-value Fisher`s Exact test. b: GG *vs*. AG+AA, *P*-value Person Chi-Square test.

Table (6) represents the distribution of genotype and allele frequencies in female diabetic group. The obese group genotyping were 47.6% (n= 10) homozygous (AA), 38.1% (n= 8) heterozygous (AG) and 14.3% (n= 3) homozygous (GG), compared to the non-obese group (control) were 39.3% (n= 11) homozygous (AA), 32.1% (n= 9) heterozygous (AG) and 28.6% (n= 8) homozygous (GG). In control group GG genotype was more prevalent as compared to other genotypes but the frequency did not significant (OR=1.34: 95% CI; 0.85-2.03, P=0.07).

Table (6): Genotypes and Allele frequencies in obese diabetic female

Genotypes	Frequencies % Control =28	Frequencies % Obese diabetic=21	*P* Value	OR (95% CI)	RR (95% CI)
AA	11 (39.3 %)	10 (47.6 %)			
AG	9 (32.1 %)	8 (38.1 %)	a 0.6	1.3 (0.37-4.25)	0.73 (0.54-2.4)
GG	8 (28.6 %)	3 (14.3 %)	b 0.07	1.34(0.85-2.03)	0.73(0.43-1.20)
Alleles					
A	31 (55.4 %)	28 (66.7 %)	0.9	1.27 (0.79-4.72)	1.76 (1.93-2.06)
G	25 (44.6 %)	14 (33.3 %)			

OR: Odds Ratio. RR: Risk Ratio. CI: Confidence Intervals. a:AA *vs*. GG+AG, *P*-value Fisher`s Exact test. b: GG *vs*. AG+AA, *P*-value Person Chi-Square test.

The significance of genetic polymorphism in LEPR gene rs1137101 for the development of obesity and type 2 diabetes mellitus has been studied in the Kurdish population from Erbil city, anthropometric and biochemical data showed that plasma leptin concentrations in obese (diabetic and non-diabetic) group increased with body mass index, this observation was generally accepted idea, since most obese individuals were leptin-resistant [13] and resistance to the actions of leptin could be caused by decreased leptin transport through the blood-brain barrier [14]. Plasma leptin levels in obese diabetics show more than two-fold increase (30.08±11.2) when compared to those of obese non-diabetic patients (12.16±1.20) and the differences was statistically significant (p=0.001), the fact that leptin inhibits insulin biosynthesis and secretion from pancreatic cells, it is also directly affects pancreatic β-cell gene expression and lead to decrease in insulin secretion [15]. Furthermore, leptin affects the β-cell mass via changes in proliferation, apoptosis, and cell growth [16] possibly explain and support our results and suggesting a role for leptin in pathogenesis of diabetes. The interesting finding of the study is a potential protective effect of GG genotype of the SNP under study against obesity in females which indicated that this genotype may associated with decrease in the risk of having obesity, more interesting is the finding that the protective effect is different between males and females of the same study group which suggests a gender specific effect, the result did not reach statistical significance but there is an obvious trend which suggest that this genotype might be protective against obesity. The lack of association of the *LEPR* gene rs1137101 polymorphism with the BMI, sex and type 2 diabetes in the whole study population is observed, and this result

is in general agreement with previous reports in Mexican population [17], United States Caucasian [18], Danese [19], British [20], and Taiwanese subjects [21]. In contrast, rs1137101 variants have been associated with these parameters of obesity in other populations [22-24].These inconsistent data could be due to ethnological factors, as well as the heterogeneity of each study population in age, sex and menstrual cycle status. The lack of association might not suggest a lack of effect; instead it reflects the complex pathogenesis of obesity which comprises environmental factors in addition to genetic component [25,26]. Large studies including testing of multiple genes in both obese and lean subjects, with epidemiologic data on dietary habits in different ethnic groups, are necessary to better understand the role of leptin in regulating weight in human populations.Thus, much work is necessary to address whether leptin effects on insulin secretion depend on glucose concentrations in human islets.

Conclusions

From the results observed, it can be concluded that the relation between diabetes and genetic variations are depended on ethnological factors and characteristic of each population. Further analysis involving a larger population is required to confirm the biological significance of rs1137101 variants in *LEPR* for obesity in the Kurdish population.

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