

## Association of the 223A/G LEPR Polymorphism with Serum Leptin Levels in Iranian Subjects with Type 2 Diabetes

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### Abstract

**Background:** Leptin, an adipocyte-derived hormone, has a pivotal role in the regulation of body weight through acting on its specific leptin receptor (LEPR). The 223A/G polymorphism of the LEPR gene is one of the most common polymorphism in all populations. In this study, we aimed to investigate the impact of the 223A/G polymorphism of the LEPR gene on serum levels of leptin in type 2 diabetes mellitus (T2DM) in a sample of Iranian population.

**Materials and Methods:** One hundred and forty-four T2DM patients were screened and compared to 147 healthy controls. The 223A/G LEPR polymorphism was genotyped using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). The serum levels of leptin were measured.

**Results:** The mean serum levels of leptin in T2DM patients were significantly higher than that of healthy control subjects; 22.90 ng/ml (95 % confidence interval [CI] = 20.79 – 25.23) vs. 8.70 ng/ml (95 % CI = 7.87 – 9.63). The genotypes (AA, AG, and GG) distributions of the 223A/G polymorphism were 55.5 %, 41 %, and 3.5 % in T2DM and 54.4 %, 42.2 %, and 3.4 % in healthy controls. The results showed no significant differences in the 223A/G LEPR genotype and allele frequencies between T2DM and control subjects ( $\chi^2 = 0.043$ ,  $P = 0.979$  and  $\chi^2 = 0.003$ ,  $P = 0.957$ ), respectively. In addition, the serum leptin levels were markedly higher in subjects with GG genotype than those with AG or GG genotype only in T2DM.

**Conclusion:** The 223A/G LEPR gene polymorphism is associated with markedly increased serum leptin levels in T2DM. However, no differences were determined in genotype and allele frequencies between T2DM patients and control subjects.

**Keywords:** LEPR, PCR-RFLP, T2DM

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### Introduction

Type 2 diabetes mellitus (T2DM), a heterogeneous and polygenic disease, is a crucial risk factor for the development of atherosclerosis, and both the prevalence and mortality of cardiovascular disease are increased in T2DM patients.<sup>1,2</sup> Leptin, an adipose tissue-derived hormone, plays a pivotal role in the control of body weight and energy balance by acting on its receptor expressed mainly in the hypothalamus.<sup>3,4</sup> The leptin receptor (LEPR) is a member of the class 1 cytokine receptor family and has a widespread tissue distribution in several alternatively

spliced isoforms in rodents and humans.<sup>5</sup> There is a growing body of evidence indicating that leptin can regulate lipid homeostasis independent of body weight changes.<sup>6-8</sup> Serum leptin level is significantly increased in obese subjects and is directly related to the body obesity.<sup>9</sup> In T2DM, leptin levels have been reported to be either unchanged,<sup>10</sup> reduced,<sup>11</sup> or increased.<sup>12</sup>

The leptin and LEPR genes have been investigated in the search for gene variants potentially related to the pathophysiology linking of obesity, T2DM, and its associated complications.<sup>13</sup> In rodents and humans it has been demonstrated that functional mutations of LEPR gene lead to the production of shorter receptor which resulted in obesity and diabetes.<sup>14-16</sup> However, such mutations are extremely rare and are not likely to be responsible for these diseases. It has been speculated that common polymorphisms in the LEPR gene through changes in the function of LEPR resulted in variations in the levels of serum leptin and body weight.<sup>17</sup>

Several common polymorphisms of the LEPR gene have been demonstrated and evaluated in populations exhibiting different prevalence rates of obesity and diabetes.<sup>18,19</sup> Among those variants, the 668 A to G transition results in change of a glutamine to an arginine at position 223 of the LEPR protein.<sup>20</sup> Moreover, the A223G polymorphism of LEPR gene has been associated with body mass index (BMI), fat mass, leptin levels, and systolic and diastolic blood pressure.<sup>21</sup> Additionally, it was reported that LEPR A223G polymorphism has been associated with impaired glu-

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cose tolerance and conversion to T2DM,<sup>22</sup> and insulin resistance.<sup>23</sup> Thus, there are few studies dealing with the association between the LEPR 223A/G polymorphism, T2DM, and obesity status. And, to date, no studies have tested the association between the LEPR 223A/G polymorphism and T2DM in the Iranian subjects. The present study was designed to investigate the influence of this common LEPR polymorphism on T2DM, serum leptin level, and lipid profile in a sample of Iranian individuals.

## Materials and Methods

### Study subjects

A total of 144 unrelated patients with T2DM and 147 unrelated non-diabetic healthy control subjects were recruited. The non-diabetic healthy control subjects were recruited from an unselected population undergoing a routine health checkup at Ahvaz Gholeshtan University Teaching Hospital. Subjects were assessed by medical history questionnaire, and fasting plasma glucose and lipid profiles were measured. The inclusion criteria for control subjects in the study were:

1. Age at least 45 years or more.
2. No past history of T2DM or of first-degree relatives with T2DM.
3. Fasting plasma glucose less than 100 mg/dl.

The diabetic subjects were randomly recruited from patients attending the outpatient's clinic of Ahvaz Gholeshtan University Teaching Hospital, Ahvaz, Iran. Diabetes was diagnosed based on the American Diabetes Association (ADA) criteria (the expert committee on the diagnosis and classification of diabetes mellitus, 1997). The study protocol was approved by the Clinical Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. Informed consent was obtained from all subjects before drawing blood. The participants in this study were assessed in the morning after at least 12-h overnight fasting. We measured weight and height of all the subjects without shoes and in light clothing. BMI was calculated as weight (kg) divided by height (m<sup>2</sup>). Blood samples were drawn for biochemical measurements. Serum total cholesterol (TC), fasting triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were measured by standardized enzymatic methods using commercial available kits (Pars Azmoon Inc., Tehran, Iran). Serum glucose was measured by a glucose oxidase method. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the equation of Friedwald, et al. for subjects with serum TG concentration of less than 400 mg/dl.

### Analysis of the 223A/G polymorphism of the LEPR Gene

Genomic DNA was obtained from whole blood by a salting out method. The 223A/G polymorphism of the LEPR gene was determined using a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The used primers were as follows: 5'-GGCCTGAAGTGTTAGAAGAT-3' (forward) 5'-CTGCTCTCTGAGGTGGGAAC-3' (reverse) and the primers information's were taken from Murugesan, et al.<sup>24</sup> Amplification was conducted in a total volume of 25 µl containing the following reagents: 2.0 µl of dNTPs (2.5 mM), 1.5 µl of MgCl<sub>2</sub> (2.5 mM), 2.5 µl of 10 × buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50ng/ µL), 0.3 µl of *Taq* polymerase (5 U/ µl), and

14.7 µl of sterile ddH<sub>2</sub>O. The amplification was performed with initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s), and extension (72 °C, 30 s), and a final extension step (72 °C, 5 min). Five µl of each PCR product, including the controls, were verified on a 2 % agarose gel ensure that the expected 642 bp product was generated. Restriction digest for the DNA fragment was carried out using *MspI* restriction enzyme. Ten µl of the PCR product was digested for 16 h overnight at 37 °C with 10 units of *MspI* (New England Bio labs). The product of the restriction digest was mixed with 1 µL of loading dye and verified on a 2 % agarose gel. The RFLP products after stained with ethidium bromide were visualized by the Avegene UV light system. The presence of an A allele at position 668 generated a unique 642 bp fragment, while the 642 bp fragment was divided into unique 173 bp and 469bp fragments when position 668 contained a G allele.

### Measurement of serum leptin level by ELISA

Serum leptin concentration was measured by ELISA using a commercially available kit (Mediagnost, Reutlingen/ Germany, E07). The inter-and intra-assay coefficients of variation were 6.8 % and 2.55 %, respectively. Briefly, first, 100 µl human leptin standards and samples were pipette into 96-well microtiter plates coated with anti leptin monoclonal antibody. After incubation at 37 °C for 60 min, the wells were washed three times and incubated at 37 °C for 30 min with the monoclonal antibody labeled with horseradish peroxidase. The wells were again washed three times and incubated with TMB reagent at 20 °C – 25 °C in dark for 15 min. Then 100 µl stop solution was added to each well, and the absorbance at 450 nm was measured.

### Statistical analysis

The clinical and biochemical parameters were presented as the mean values ± SD (standard deviation) and as percentage for categorical variables. Variables that were not distributed normally were log-transformed. Comparisons between T2DM patients and healthy control subjects were made using unpaired *t*-test. All frequencies were estimated by gene counting. A goodness-of-fit test  $\chi^2$  test was used for evaluation of the Hardy-Weinberg equilibrium. Also,  $\chi^2$  tests were used to compare genotype distribution and allele frequencies and other quantitative data. Significant differences between the anthropometric and metabolic characteristics of different LEPR genotypes were tested by one-way analysis of variance (ANOVA) with Tukey's test for post hoc comparisons of each group. A *P*-value < 0.05 was considered to be statistically significant for all analyses. All statistical analyses were performed with SPSS software version 15.

## Results

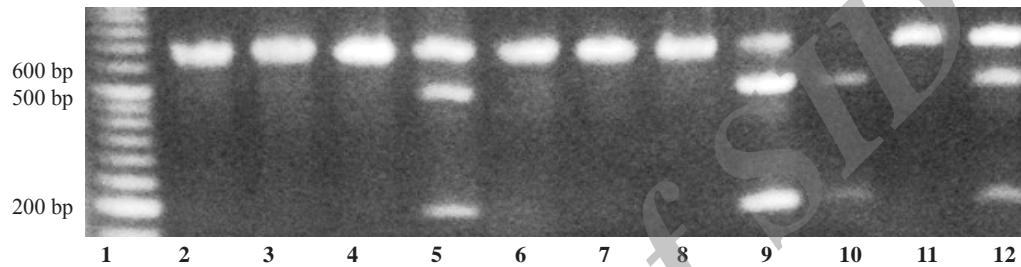
### Anthropometric and biochemical characteristics of two groups

The anthropometric and biochemical characteristics of T2DM patients and healthy control subjects are presented in Table 1. Compared to healthy control subjects, T2DM patients had significantly higher concentrations of TC (*P* = 0.022), TG (*P* < 0.001), LDL-C (*P* = 0.049), and fasting glucose (*P* < 0.001), whereas these patients had significantly lower averages of HDL-C (*P* < 0.001). In addition, serum leptin levels in patients with T2DM were significantly higher than those in healthy control individuals (*P* < 0.001).

**Table 1.** Biochemical and anthropometric characteristics of T2DM and control subjects

Variables	control (n = 147)	T2DM (n = 144)	P- value
Gender (M/F, %)	43/57	40/60	0.571
Age (years)	52.53 ± 7.31	54.33 ± 8.85	0.060
Height (cm)	163.91 ± 9.31	163.25 ± 9.33	0.549
Weight (kg)	75.48 ± 13.73	73.20 ± 12.20	0.136
BMI (kg/m <sup>2</sup> )	28.02 ± 4.01	27.6 ± 4.50	0.439
Total cholesterol (mg/dl)	181.84 ± 39.31	194.06 ± 50.23	0.022
Triglyceride (mg/dl) <sup>a</sup>	134.90 (126.15; 144.24)	165.95 (154.17; 178.64)	< 0.001
LDL-C (mg/dl)	103 ± 40.73	113 ± 46.04	0.049
HDL-C (mg/dl) <sup>a</sup>	46.77 (45.40; 48.18)	41.68 (40.45; 42.95)	< 0.001
Fasting glucose (mg/dl) <sup>a</sup>	91.20 (86.25; 96.42)	169.82 (159.91; 180.34)	< 0.001
Leptin (ng/ml) <sup>a</sup>	8.70 (7.87; 9.63)	22.90 (20.79; 25.23)	< 0.001

Number of individuals in parentheses. Continuous variables are presented as mean ± SD and were compared by independent sample *t*-test. Categorical variables were compared by  $\chi^2$ . *P* < 0.05 was considered as significant. Natural logarithmic transformations were performed before analysis; <sup>a</sup> geometric mean with 95 % confidence intervals in parentheses.

**Figure 1.** Representative agarose gel electrophoresis picture of PCR-RFLP product for the 223A/G LEPR. Lane 1 shows 50bp DNA ladder (CinnaGen.Co); lanes 2, 3, 4; lanes 6,7,8; and lane 11 show wild type genotype AA (642bp); , lane 5, lane 9, and lane 12 show heterozygous genotype AG (642, 469, 173 bp); lane 10 shows homozygous mutant GG (469, 173 bp).

#### Association of the LEPR 223A/G polymorphism with T2DM

After agarose gel electrophoresis, genotypes were determined by comparing the length of the restriction fragment for the 223A/G LEPR polymorphism. Three genotypes identified: subjects with AA genotype (wild-type) showed one single band of 642 bp; heterozygous with AG genotype showed three bands of 642 bp, 469 bp, and 173 bp; and homozygous carrying the GG genotype showed two bands of 469 and 173 bp (Figure 1). The genotypes distribution and allele's frequencies of the 223A/G LEPR polymorphism in T2DM patients and healthy control subjects are presented in Table 2. The genotype frequencies were as follows: 55.5 % (AA), 41 % (AG), and 3.5 % (GG), in T2DM patients and 54.4 % (AA), 42.2 % (AG), and 3.4 % (GG) in healthy control subjects. In addition, the frequencies of alleles A and G were: 76 % and 24 % in T2DM patients, and 75.9 % and 24.1 %, in healthy control subjects. The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium in T2DM patients and healthy control subjects. The genotype and allele frequencies of the 223A/G LEPR polymorphism were similar between healthy control subjects and T2DM patients (*P* > 0.05).

#### Association of biochemical parameters and the LEPR 223A/G polymorphism

In healthy control subjects, among three genotypic subgroups, there were no significant differences in age, BMI, TC, TG, HDL-C, LDL-C, serum glucose, and serum leptin levels (Table 3). In T2DM patients, subjects having heterozygous AG variant had significantly higher HDL-C than those with AA homozygotes (*P* <

0.05), whereas the other parameters, including, TC, serum glucose, and LDL-C were no significantly different (*P* > 0.05) (Table 3).

#### Influence of the 223A/G polymorphism of the LEPR gene on serum leptin levels

To determine whether the 223A/G polymorphism of the LEPR gene is associated with differences in serum leptin levels, we measured serum leptin by ELISA method. Although, no significant differences in leptin screened across genotypes were found in the control group. However, T2DM patients carrying the GG genotype exhibited higher serum leptin levels compared to those with AA and AG genotypes (*P* = 0.002, *p* = 0.024), respectively (Table 3).

## Discussion

In this study, we reported no significant differences in genotypic distribution and allele frequencies of the 223A/G LEPR polymorphism between T2DM patients and healthy control subjects, in a sample of Iranian population. Additionally, we found no association of the 223A/G LEPR polymorphism with anthropometric indices (weight, height, and BMI) and metabolic characteristics in T2DM patients and healthy control subjects. However, we observed a significant association between the 223A/G LEPR polymorphism and higher leptin concentrations in T2DM patients. Additionally, there was a significant difference in HDL concentration between genotypes of the 223A/G LEPR in T2DM patients.

Although the role of 223A/G polymorphism of the LEPR gene has been assessed in previous studies, but its impacts on T2DM patients have not been completely understood. LEPR is a single

**Table 2.** Genotypes distribution and allele frequencies of the 223A/G LEPR in T2DM and control subjects

Group	Genotype			Allele	
	AA (%)	AG (%)	GG (%)	A (%)	G (%)
Control (n = 147)	80 (54.4 %)	62 (42.2 %)	5 (3.4 %)	223 (75.9 %)	71 (24.1 %)
T2DM (n = 144)	80 (55.5)	59 (41)	5 (3.5)	219 (76)	69 (24)
	$\chi^2 = 0.043$ ; P = 0.979			$\chi^2 = 0.003$ ; P = 0.957	

Data are presented as number of cases, with frequency in parentheses. Allele frequencies and genotypes distribution of the 223A/GLEPR in T2DM patients were compared to those in control subjects.

**Table 3.** Characteristics of the study population according to the 223A/G LEPR genotypes

	Control			T2DM		
	AA	AG	GG	AA	AG	GG
Age (year)	52 ± 7	52 ± 7	56 ± 6	54 ± 8	54 ± 9	49 ± 5
BMI (kg/m <sup>2</sup> )	28.2 ± 4.3	27.8 ± 3.4	27.2 ± 5.8	28.3 ± 4.4	26.5 ± 4.2	30.0 ± 4
TC (mg/dl)	183.4 ± 41	180.2 ± 35	175.4 ± 39	191.6 ± 47	198.2 ± 54	183.2 ± 50
TG (mg/dl) <sup>a</sup>	134.9 (122.5; 148.4)	141.2 (127.4; 156.6)	112.2 (63.2; 199.0)	165.9 (116.5; 236.2)	162.1 (142.5; 184.5)	181.9 (68.70; 481.9)
LDL-C (mg/dl)	105.9 ± 41	101.3 ± 38	100.92 ± 58	111.7 ± 43	118.5 ± 50	93.0 ± 22
HDL-C (mg/dl) <sup>a</sup>	45.7 (29.0; 47.8)	47.8 (47.0; 48.6)	44.6 (36.55; 54.57)	40.7 (39.3; 42.1)	44.6 (43.8; 45.4)	40.73(34.27; 48.41)
FBS(mg/dl) <sup>a</sup>	89.1 (80.5; 98.5)	93.3 (89.1; 97.7)	87.09 (79.98; 94.84)	165.9 (157.7; 174.5)	169.8 (160.1; 180.0)	169.8 (127.6; 225.94)
Leptin (ng/ml) <sup>a</sup>	8.91 (7.46; 10.63)	7.76 (6.46; 9.32)	22.38 (11.91; 42.07)	19.95 (16.3; 24.41)	25.11 (14.8; 42.61)	63.09 (22.49; 177.0)**

Data are means ± SD. Differences between three genotypes were examined by one-way analysis of variance (ANOVA) with Tukey's test for post hoc comparisons of each group. Results were presented for the dominant genetic model (homozygous for 223-AA vs. carrier of G allele). Natural logarithmic transformations were performed before analysis. Compared with the AA and AG genotypes, \*\*P < 0.05. Compared with the AA genotype, \*P < 0.05; <sup>a</sup> geometric mean with 95 % confidence intervals in parentheses. BMI: Body mass index; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein; HDL-C: High-density lipoprotein; FBS: Fasting blood sugar.

trans- membrane domain receptor with two cytokine domains, which binds to leptin.<sup>25</sup> In Zucker fatty (*fa/fa*) rats, a missense mutation (an A to C conversion at nucleotide position 806) in the extracellular domain of the LEPR gene results in a single amino acid change from Q to P, at position 269 of LEPR protein.<sup>26</sup> The *fa* mutation gives rise to an abnormal LEPR that is unable to constitutively activate the signal pathway and is highly impaired for ligand-induced activation.<sup>27</sup> Since both the 223A/G polymorphism and the *fa* mutation occur in the first cytokine domain, it is highly possible that the 223 A/G polymorphism could also affect the function of LEPR. Furthermore, the conservation of this amino acid among the human, the rat, and the mouse propose the possibility of a functional importance of this amino acid in the LEPR.

Our results showed that there was a significant association between AG genotype of the 223A/G LEPR and higher HDL-C concentration only in T2DM patients. A number of studies in mice and humans demonstrated that leptin plays a regulatory effect on the metabolism of HDL-C.<sup>28,29</sup> It has been proposed that leptin could be effective on the gene expression of hepatic lipase and several Apo lipoprotein genes.<sup>30</sup> It is speculated that the 223A/G polymorphism of the LEPR gene was related to the lower binding capacity of leptin to its soluble receptor.<sup>31</sup> On the other hand, a study in postmenopausal Caucasian women demonstrated that leptin binding capacity to its soluble receptor was reduced in the subjects carrying A allele of the 223A/G polymorphism.<sup>31</sup> Another study in Pima Indians found that individuals carrying AA genotype have significantly lower energy expenditure and physical activity compare to the AG and GG genotypes.<sup>5</sup> It is suggested that leptin or A allele of the 223A/G polymorphism of the LEPR gene through changes in the activity of hepatic lipase, phospholipid

transfer protein, cholesteryl ester transfer protein, or lipoprotein lipase may contribute to the decreased serum levels of HDL-C.<sup>31</sup> In addition, univariate linkage analyses in hypertensive sib ships demonstrated a locus for HDL-C levels on chromosome 1, which also contain the LEPR gene.<sup>32</sup> In general, the 223A/G polymorphism of the LEPR gene may contribute to the changes in lipid profile through leptin signaling pathway. And it is speculated that there is a relationship between the 223A/G polymorphism of the LEPR gene and HDL-C metabolism.

Our results revealed that the 223A/G polymorphism of LEPR gene affected serum leptin concentration only in T2DM patients. The relationship between leptin level and the LEPR 223A/G polymorphism are conflicting. Murugesan, et al. has reported that the presence of the 223G allele in the GG genotype form was significantly associated to the BMI, insulin, and leptin levels. Also, they revealed that GG genotype form is a significant predictor of T2DM in the studied population. Also, they speculated that the polymorphism A223G may act as a strong marker in the local population of Coimbatore.<sup>24</sup> Yiannakouris, et al. have demonstrated that carriers of the LEPR 223G allele had significantly higher leptin level than non-carriers.<sup>33</sup> Constantin, et al. reported that in the Romanian populations, subjects carrying the G allele exhibited higher serum leptin than those homozygous for A allele.<sup>34</sup> The other previous studies found positive associations of the G allele with high leptin levels in healthy populations<sup>35</sup> or in patients with insulin resistance phenotypes.<sup>23</sup> In contrast, Quinton, et al. have indicated that the LEPR 223 G allele was associated with lower circulating leptin level.<sup>31</sup> Additionally, Ben Ali, et al. have showed that obese male patients carrying the G allele had significantly lower leptin levels than subjects homozygous for the A allele. And

in obese women, the 223A/G LEPR polymorphism was found to be associated with lower leptin concentrations.<sup>36</sup> Several factors are now considered as possible mediators and regulators of serum leptin levels. It is demonstrated that serum levels of leptin are highly associated with BMI and body fat mass.<sup>37</sup> Moreover, generally women have higher circulating levels of leptin compared to men.<sup>38</sup> Insulin stimulates leptin synthesis, whereas thyroid hormones inhibit leptin secretion.<sup>39,40</sup> Another mechanism is that changes in the sequence of gene coding for LEPR may affect the expression of this protein. A study in postmenopausal Caucasian women revealed that the 223A/G polymorphism of the LEPR was associated with lower leptin levels.<sup>31,40</sup> This finding was confirmed by a study in Mexican adolescents.<sup>41</sup> Inversely, in the current study, the 223A/G polymorphism, especially the G allele, is associated with higher serum leptin levels in T2DM patients but not in healthy control subjects.

In summary, we did not find any specific genotype in the 223A/G LEPR gene polymorphism to be associated with T2DM in a sample of Iranian population. Also, we found that the 223A/G polymorphism of the LEPR gene affected the serum leptin concentration in T2DM patients. This is the first report demonstrating that the 223A/G polymorphism of the LEPR gene influences on serum leptin levels and no association of this polymorphism with T2DM patients in Iranian subjects. A carefully designed study is required to examine the functional consequence of the 223A/G polymorphism in T2DM patients.

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