ORIGINAL ARTICLE

Association between leptin gene G-2548A polymorphism with metabolic syndrome

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Background: Metabolic syndrome (MetS) is a serious public health problem. It is an important risk factor of cardiovascular disease in developed countries. Adipose tissue considered as an organ that releases a variety of molecules referred to adipocytokines such as leptin.Polymorphism of their related genes may play an important role in development of MetS. The aim of this study was to determine the association of leptin gene-2548G/A (LEP-2548G/A) polymorphism with lipid profile in subjects with and without Mets. **Materials and Methods:** In this case/control study a frequency of LEP-2548G/A single nucleotide polymorphism was determined between 200 patients (142 women and 58 men) and 200 controls (122 women and 78 men). Both groups were selected randomly from Hamadan city, Iran. Blood samples were collected then followed by routine biochemical analysis, DNA extraction and serum leptin measurements. Polymerase chain reaction-restriction fragment length polymorphism was applied to identify LEP-2548G/A genotypes. Statistical analyses were applied using SPSS software version 10. Continuous variables were presented as means± SD and compared by independent sample t-test. Variables without normal distribution compared through Mann-Whitney U test. **Results:** In both groups, a significant difference was observed between biochemical factors and leptin concentration. Serum leptin concentration was more in females than males. No statistical significant difference was detected in the frequency of LEP-2548G/A polymorphism between both MetS and healthy groups. **Conclusion:** In summary, it is concluded that frequency of LEP G-2548A polymorphism in Metabolic syndrome (MetS) and healthy subjects was not significantly different and more research with large sample size is needed in this area.

Key words: Leptin, metabolic syndrome, -2548G/A polymorphism

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INTRODUCTION

Metabolic syndrome (MetS) includes a clustering of multiple cardiovascular risk factors as abdominal obesity, hypertension, insulin resistance and dyslipidemia.[1] This syndrome is an epidemic and public health problem in the developed world. The etiology of the MetS includes environmental factors, genetic susceptibility, and their interactions.[2] It is believed that central obesity is the main symptom of MetS.[3] Adipose tissues are an endocrine organ which secretes various adipokines. These tissues participate in the regulation of metabolic and inflammatory processes related to abdominal obesity.[4] On the other hand, a high number of single nucleotide polymorphisms (SNPs) have been identified in different genes as cholesteryl ester transfer protein (CETP), leptin, apoA1 and apoE. The products of these genes affects on the risk factors of MetS. The other studies have been shown that occurring of SNPs in CETP gene may cause a change in lipid profile and CETP activity and concentration. This mechanism may lead to hyperlipidemia.[5-8]

Leptin, a 16-kDa polypeptide hormone plays an important role in several systemic effects such as body mass control, fertility, angiogenesis, immunity, and hematopoiesis. Also its key role in food intake and energy balance is very important. This may affect on the onset of obesity and other systemic defects like MetS.[9-13] Leptin concentration is dependent on the fat body mass and is higher in women than men.[9] Though, it is markedly elevated in obese individuals, but people with a similar adiposity show great variations in leptin concentrations.[10,14]

The occurrence of SNP in the non-coding region of the leptin gene (LEP) may cause different concentrations of leptin and this may play a role in human obesity.[15-18] Some studies suggested that variations in translated part of the LEP cannot describe widespread obesity.[16] Some variants of SNPs in the 5′ region flanking of LEP have been detected.[15,19] 2548G/A leptin promoter SNP is a common mutation which possibly to be associated with serum leptin concentration or body mass index (BMI).[15-19]

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Therefore, it was hypothesized that the LEP-2548 G/A polymorphism could be associated with the pathogenesis and severity of MetS. Based on our knowledge, there are no other studies available to explore the frequency of sequence diversity in the regulatory region of this gene and its relation with MetS. The aim of this study was to determine the association of LEP-2548G/A polymorphism with lipid profile in subjects with and without MetS.

SUBJECTS AND METHODS

Study subjects

The study group composed of volunteers: 200 subjects (58 men and 142 women) met the MetS criteria, the other 200 subjects (78 men and 122 women) were considered as the healthy group. Anthropometrics measurements such as BMI and waist circumference (WC) were taken from each participant. Systolic/diastolic blood pressure was measured in sitting position following resting for 5 min. Inclusion criteria for MetS patients were based on modified criteria from the National Cholesterol Education Program-Adult Treatment Panel III.[20] According to this criteria patients with three or more of the following conditions were included in the study: (a) central obesity (WC > 90 cm); (b) high blood pressure ≥ 130/85 mmHg or documented use of antihypertensive therapy; (c) high fasting glucose (≥100 mg/dL); (d) hypertriglyceridemia (≥150 mg/dL), and (e) low-high density lipoprotein cholesterol (<50 mg/dL). Those patients who were under estro-progesterone or testosterone treatment and those that were taken contraceptive and diuretic drugs and also pregnant females were excluded from this study. This study was approved by the Hamadan University of Medical Sciences Ethical Committee and informed consent was obtained from each participant.

Biochemical methods

A 12-h fasting blood sample was obtained from each participant. Glucose, triglycerides, total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were measured by enzymatic-colorimetric assays (Pars-Azmoon Company, Iran). Low-density lipoprotein cholesterol (LDL-C) was calculated by Friedewald formulae. Plasma leptin was determined by enzyme linked immuno sorbent assay method (DIAsource, Belgium).

DNA extraction and detection of leptin gene-2548G/A polymorphism

Genomic DNA was extracted from 1 mL Ethylenediaminetetraacetic acid (EDTA)-anti coagulated whole blood using DNA purification (DNP)™ kit (Cinagen, Iran). Polymerase chain reaction (PCR)-restriction fragment length polymorphism strategy was applied to detect-2548G/A polymorphism using *Hha1 (Cfo1)* restriction enzyme (Fermentas, USA). The primers used in PCR assays were designed by Allele ID6. The forward primer (5′-ttt cct gta att ttc cca tga g-3′) and the reverse primer (5′-aaa gca aag aca ggc ata aaa-3′) that generates a PCR product (242 bp from nucleotides -2392 to -2634) with a constitutive site for *Hha1*.The polymorphism was defined by presence (G allele) or absence (A allele) of a *Cfo*I restriction site that cut the sequence gcgc in the second G site. Excision will happen in the case of wild type and the result is two fragments with 181 and 61 bp length. The PCR reaction was carried out in a total volume of 50 μl containing 10 μg genomic DNA, 0.2 pmol/L of each primer (Bioneer, Korea), 200 μmol/L dNTPs, 2.5 U Taq DNA polymerase, 50 mM KCl, 1.5 mmol/L $MgCl_2$, and 10 mmol/L Tris-HCl pH 8.4. An Eppendorf thermal cycler was applied to perform PCR reaction. The used program was: Initial denaturation at 96°C for 10 min followed by 30 cycles of amplification, each cycle consisting of 30 s denaturation at 96°C, 30 s annealing at 50°C and 30s extension at 72°C. The reaction ended with an additional 7 min of extension at 72°C. The quality of PCR products was evaluated in a 2% agarose gel electrophoresis stained with syber safe. PCR products were incubated with 4 U of *Hha1* at 37°C for 15 min. Digested product was separated on 15% polyacrilamide gel electrophoresis at 120 V for 8 h and analyzed under ultra-violet light following ethidium bromide staining. Accuracy of the genotyping was evaluated by performing duplicate analysis of 30% of randomly selected samples. Furthermore, a confirmed heterozygous LEP-2548G/A sample was included during each electrophoresis as a positive control.

Statistical analysis

Statistical tests were performed using SPSS software version 10. Continuous variables were presented as means ± SD and compared by independent sample *t*-test. Variables without normal distribution were compared through Mann-Whitney U test. Genotype frequencies were analyzed applying Hardy-Weinberg equilibrium expectations and Chi-square test. Pearson correlation coefficient was used to determine the relationship between continuous variables. Chi-square test was performed for comparing qualitative variables between two groups. Kolmogorov-Simonov goodness of fit test was performed to evaluate normality of quantitative variable. Since, the test shows the studied variables are normally distributed, for comparing the quantitative variables in the two studied groups; independent sample *t*-test was used. *P* value less than 0.05 was considered as a statistically significant.

RESULTS

Anthropometric and clinical parameters of all the participants are presented in Table 1. The patients with MetS were 58 men (29%) and 142 women (71%) with a mean age of 44.3 ± 13.5 years old. The control group was consisted of 78 men (39%) and 122 women (61%) with a mean age of 37 ± 12.5 years old. No statistically difference was observed between the MetS patients and control group based on the

sex, smoking and family history of illness. In both study and control groups, higher concentration of plasma leptin was observed in females compared to males ($P < 0.05$). In MetS group, a statistically difference between men and women was explored according to TC, HDL-C and LDL-C concentrations. Furthermore, in non-MetS group, there was a significant difference between women and men based on Triglyceride and WC (data not shown).

Comparison of all the mentioned variables in men or women members of both Mets and control groups were shown a significant difference ($P < 0.01$) [Table 2] except diastolic pressure in women.

In both MetS and control groups, genotype distribution and allele frequencies for LEP-2548 G/A polymorphism have been presented in Table 3. The-2548G/A polymorphism was common among the study group and the-2548A allele was the more frequent allele with a frequency of 58.55% [Table 3]. There was no significant difference between two groups and also between genders according to genotype and allele frequencies. The distribution of genotypes observed in both the control and case samples were in Hardy-Weinberg equilibrium.

Table 1: Clinical and biochemical characteristics of study groups

	MetS	Non-MetS	*P* value
BMI (kg/m^2)	30.5±5.7	26.8±5.8	<0.05
Waist circumference (cm)	100.8±11	90.5±14.8	<0.05
Systolic pressure (mmHg)	12.7±1.8	11.5±1.4	<0.05
Diastolic pressure (mmHg)	8.3±1.1	7.7±0.8	<0.05
Fasting glucose (mg/dL)	105.3±36.3	89.5±13.4	<0.05
Total-cholesterol (mg/dL)	202±42	177.6±33.3	<0.05
Triglycerides (mg/dL)	194.9±80.8	129±48	<0.05
HDL-C (mg/dL)	41.6±10.5	46.5±8.3	<0.05
LDL-C (mg/dL)	123±35.5	102.4±30.5	<0.05
Leptin (ng/ml)	19.4±15	10.3±8.3	0.001

TC=Total cholesterol; TG=Triglyceride; HDL-C=High-density lipoprotein cholesterol; LDL-C=Low-density lipoprotein cholesterol; MetS=Metabolic syndrome

It was detected a borderline significant difference in control`s systolic pressure ($P = 0.054$) and also in MetS group's BMI, waist and TC in those with 2548G/A genotypes ($0.05 < P < 0.08$). Only in non-MetS group, Fasting Blood Sugar (FBS) and LDL-C showed significant difference between genotypes with a P value of 0.005 and 0.043 respectively. In AA genotype, comparison of parameters between genders showed a higher value of LDL-C ($P = 0.018$), BMI ($P = 0.014$) and leptin concentration ($P = 0.005$) in females.

Mean concentration of leptin based on the genotypes has been presented in Table 4. Those subjects with mutant allele in their genotype got higher leptin concentration. Also MetS patients got more concentrations of leptin than control subject in all genotypes. Leptin concentration is associated with some other parameters that were shown briefly in Table 5.

DISCUSSION

A common single nucleotide polymorphism within the 5′ promoter region of leptin gene is G-2548A. This polymorphism has been reported to be associated with leptin production and BMI in obese individuals.[10,15,16,18]

It is shown that leptin is necessary for induction and maintenance of the proinflammatory Th1 immune response.[13,21] Also it is known that chronic inflammation play a key role in MetS. In this study, in a population of MetS patients, a relationship between leptin levels and abdominal obesity was evaluated. Concentration of leptin in the serum is strongly correlated with body fat mass in healthy animals and humans.[10,14] Moreover, females compared with males have higher leptin concentrations.[22] In this study it was reported that leptin concentration is higher in females in both groups. Also it was higher in MetS groups than the control group. Previous studies have shown both increased[16,17] and decreased[18] concentrations of circulating leptin in those that carry A/A genotype. No significant correlation was observed in different genotypes according to leptin concentrations.

Table 2: Characteristics comparison between women and men of study groups

	Women			Men		
	MetS	Non-MetS	*P* value	MetS	Non-MetS	*P* value
BMI (kg/m^2)	30.2±6.7	26.3±5.7	<0.001	30.5±5.3	27±5.8	<0.001
Waist circumference (cm)	103±11.8	93.3±12.7	<0.001	100±10	88.8±15.8	<0.001
Systolic pressure (mmHg)	14.4±1.2	11.7±1	0.049	12.6±1.7	11.4±1.6	<0.001
Diastolic pressure (mmHg)	9.8±0.7	7.8±0.6	NS	8.8±0.6	7.6±0.8	NS
Fasting glucose (mg/dl)	112±33.8	90.7±16	<0.001	102.7±37	88.6±11.3	<0.001
TC (mg/dl)	191.8±35.3	181.5±36.6	NS	206±44	175±30.8	<0.001
TG (mg/dl)	198.8±81	145±51.4	<0.001	193±80	118.7±42.6	<0.001
HDL-C (mg/dl)	38.7±5.3	45.6±10.3	<0.001	42.7±11.8	47±6.7	<0.001
LDL-C (mg/dl)	111.3±30.8	101.6±31.6	NS	127.6±36.3	103±30	<0.001
Leptin (ng/ml)	15.3±11.7	8.8±7.4	<0.001	21±15.7	11.3±8.7	<0.001

BMI=Body mass index; TC=Total cholesterol; TG=Triglyceride; HDL-C=High-density lipoprotein cholesterol; LDL-C=Low-density lipoprotein cholesterol; NS=Not significant; MetS=Metabolic syndrome

Table 3: Genotype distributions and allele frequencies of the leptin gene G-2548A polymorphism in MetS and non-MetS subjects

	MetS (%) (*n**)	Non-MetS (%) (*n**)	*P* value
GG	8.29 (18)	15 (30)	NS
GA	66.32 (131)	58.5 (117)	NS
AA	25.39 (51)	26.5 (53)	NS
G	0.415	0.423	NS
A	0.585	0.577	NS

NS=Not significant; *Values in parenthesis are number of subject with genotype; MetS=Metabolic syndrome

Table 4: Mean±SD of leptin concentration (ng/ml) classified based on genotype

Groups	Non-MetS	MetS
GG	11.6±8.7	16.3±12.8
GA	10±8	18.9±13.8
AA	10.2±8.2	21.8±17.9
P value	0.677	0.334

MetS=Metabolic syndrome

Table 5: Pearson correlation of leptin concentration with other parameters

Parameters	*P* value	*R*
TG	0.016	0.121
TC	0.007	0.136
HDL-C	0.020	−0.118
LDL-C	0.001	0.160
Waist	0.035	0.106

TC=Total cholesterol; TG=Triglyceride; HDL-C=High-density lipoprotein cholesterol; LDL-C= Low-density lipoprotein cholesterol

However, in control and MetS groups, those with GG and AA genotypes had higher leptin concentrations respectively. Hoffstedt *et al*.[19] reported non-obese women with the A/A genotype had higher serum leptin concentrations. However, in this study no difference was observed in leptin concentration of females with A/A genotype in both MetS or non-MetS groups.

The other studies[16] reported males who are homozygous for allele A had higher leptin concentrations. However, this study did not detect any difference in serum leptin in those with A/A genotype. In contrast to our results, Le Stunff *et al*.[20] reported association between this polymorphism and leptin concentrations. According to that study, females with the A/A genotype have lower leptin concentrations compared to those with G/A and G/G genotypes.

Frequency of LEP-2548G allele (41.85%) that observed in subjects from Hamadan of Iran was not similar to those which found in Europeans,[16,18] North Americans[23] and Brazilian[24] populations. Although Le Stunff *et al*.[18] showed a comparable genotype distribution in obese girls with normal weight; our results did not show any significant difference in genotype distribution between MetS and control groups.

Data on association of this polymorphism with obesity, BMI and fat mass is controversial. These different results may be due to interactions of this polymorphism with other parts of leptin and/or leptin receptor genes. Furthermore, gender and genetic background of Iranian population may be the other explanation for these challenging results. In Tunisian obese and normal weight groups, no association was observed between LEP-2548G/A polymorphism and obesity. Furthermore, in this mentioned study, no significant differences was shown in mean of BMI, insulin and plasma lipid concentrations among the G-2548A LEP genotypes in both obese and normal weight subjects.[25]

Furthermore, in Romanian population, no association was found between the LEP-2548G/A polymorphism and obesity and obesity-related variables as BMI, fat mass, WC, and WHR. However, in this study a higher leptin concentration was identified in-2548GG carriers.[26] On the other hand, Li *et al*. reported an association of this polymorphism with extreme obesity in North American Caucasian women,[19] in whom the G allele was more frequent.

Several factors, other than gender and body fat could be considered as responsible for individual variation of leptin concentrations. In this respect, it has been reported that insulin is a modulator of the leptin production.[27] Obesity increases the risk of type 2 diabetes. In another study, a hyperleptinemia and decrease sensitivity to insulin has been reported in obese subjects that ultimately this lead to diabetes.[28] Moreover, fat mass, leptin transcription and its synthesis can be induced by high concentrations of insulin.[29] On the other hand, one of the aspects of MetS is insulin resistance and hyperinsulinemia. So it was hypothesized these hyperinsulinemia may play a role to increase concentration of leptin in MetS group. As the LEP-2548G/A site in the leptin promoter is proximal to Sp1 binding motif[30] and nucleolin could interact with Sp1,[31] existence of A allele in Lep-2548 might enhance insulin-dependent activation of the leptin promoter by promoting the interchange between transcriptional regulators like nucleolin as negative and Sp1 as positive regulators.[31-33]

Effects of the G to A substitution at the position of 2548 and its role in leptin expression remain to be elucidated.

Our data do not support strong association of the LEP-2548G/A with MetS. Positive correlations between leptin and lipids concentrations suggest its possible role as a risk factor for Coronary Artery Disease (CAD) . Leptin applies many potentially atherogenic effects such as stimulation of endothelial dysfunction, induction of inflammatory reaction, oxidative stress, platelet aggregation and proliferation of vascular smooth muscle cells.[34] These consequences may contribute to the pathogenesis of some obesity disorder like hypertension and atherosclerosis.[35,36]

In summary, we have shown that the-2548G/A polymorphism in the promoter region of the human leptin gene was not associated with serum leptin concentration in both MetS and healthy groups. Although this study point to no significant difference between frequency of MetS and non-MetS genotype (A/A, A/G and G/G), however it shows a correlation of leptin concentration with lipid concentration and WC. In order to discover effects of this polymorphism on leptin and its relation with obesity more research is needed.

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