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Leptin G-2548A Promoter Polymorphism is Associated with Increased Plasma Leptin and BMI in Brazilian Women

ABSTRACT

Variants in leptin gene (*LEP*) have been implicated in the pathogenesis of obesity. The relationship between *LEP* G-2548A polymorphism and obesity-related traits was evaluated in a sample of Brazilian women (n = 228) who were randomly selected from two clinical centers in Sao Paulo city. Blood samples were collected for DNA extraction, plasma leptin and serum lipids measurements. *LEP* G-2548A genotypes were identified by a PCR- RFLP strategy using the endonuclease *Alw*44I. *LEP* G-2548A was associated with obesity after adjustment for covariates (age, hypertension, coronary artery disease, smoking and physical activity). Women carrying G allele had a four times higher risk of obesity than the A allele carriers (OR: 4.11, CI95%: 1.06-15.90, *p* = 0.041). G allele was also related to increased plasma leptin (p = 0.024) and body mass index (p = 0.027). Hypertension, hyperglycemia, dyslipidemia and coronary artery disease were associated with obesity. However *LEP* G-2548A polymorphism was not related to these variables. All together these data suggest that *LEP* G-2548A polymorphism has an important role in regulating plasma leptin levels and body mass index in women. **(Arq Bras Endocrinol Metab 2008; 52/4:611-616)**

Keywords: Leptin; Gene polymorphism; Body mass index; Obesity; PCR-RFLP.

RESUMO

Polimorfismo do Promotor do Gene da Leptina está Associado ao Aumento de Leptina Plasmática e IMC em Mulheres Brasileiras.

Variantes no gene da leptina (*LEP*) foram implicados na patogênese da obesidade. A relação entre o polimorfismo *LEP* G-2548A e as características relacionadas com a obesidade foram avaliadas em mulheres brasileiras (n = 228), que foram selecionadas randomicamente de dois centros de pesquisa clínica na cidade de São Paulo. As amostras de sangue foram coletadas para extração de DNA e determinações de leptina plasmática e lipídeos séricos. Os genótipos do *LEP* G-2548A foram identificados pela estratégia de PCR-RFLP, empregando a endonuclease *Alw*44I. O polimorfismo *LEP* G-2548A foi associado com obesidade, após ajuste para as covariáveis: idade, hipertensão, doença arterial coronariana, tabagismo e atividade física. Mulheres com alelo G tiveram quatro vezes maior risco de obesidade que as portadoras do alelo A (OR: 4,11, CI95%: 1,06-15,90; *p* = 0,041). O alelo G também foi relacionado com leptina plasmática (p = 0,024) e o índice de massa corporal (p = 0,027) aumentado. A hipertensão, a hiperglicemia, a dislipidemia e a doença arterial coronariana foram associadas com obesidade. Entretanto, o polimorfismo *LEP* G-2548A não foi relacionado com essas variáveis. Os resultados deste estudo são sugestivos de que o polimorfismo *LEP* G-2548A tem papel importante na regulação da leptina plasmática e no índice de massa corporal em mulheres. **(Arq Bras Endocrinol Metab 2008; 52/4:611-616)**

Descritores: Leptina; Polimorfismo genético; Índice de massa corporal; Obesidade; PCR-RFLP.

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HAMILTON M. HINUY
MARIO H. HIRATA
NEUSA FORTI
JAYME DIAMENT
MARCELO F. SAMPAIO
DIKRAN ARMAGANIJAN
LUIS A. SALAZAR
ROSARIO D. C. HIRATA

Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (USP), SP, Brasil (HMH, MHH, RDCH); Instituto do Coração da Faculdade de Medicina (InCor-FM) da USP, São Paulo, SP, Brasil (NF, JD); Instituto Dante Pazzanese de Cardiologia de São Paulo, SP, Brasil (MFS, DA); Facultad de Medicina da Universidad de La Frontera, Temuco, Chile (LAS).

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INTRODUCTION

The prevalence of obesity is increasing in most of industrialized countries and has become an important issue of public health. Excessive bodyweight is considered the sixth most important risk factor contributing to the overall burden of disease worldwide (1). Obesity has a complex pathogenesis that results from interactions between genetic and environmental factors that lead to the malfunction of several signaling peptides, which are involved in body energy balance and nutritional status (2,3).

Leptin is a metabolic and neuroendocrine hormone produced and released mainly by adipocytes (4,5). It has several systemic effects such as body mass control, reproduction, angiogenesis, immunity, wound healing, bone remodelling and cardiovascular function (4,6). Plasma leptin concentration is proportional to body adiposity and is markedly increased in obese individuals (4).

Several studies have suggested that variants in the leptin gene (*LEP*) may be important to the pathophysiology of human obesity (7,8). A common single nucleotide polymorphism within the 5' promoter region (G-2548A) of *LEP* has been associated with variations in plasma leptin and body mass index (BMI) in obese individuals (9-11). It has been shown that the G-2548A *LEP* polymorphism influences leptin expression, possibly at the transcriptional level, and therefore also adipose secretion levels of the hormone (12).

In this study, the influence of the *LEP* G-2548A polymorphism on plasma leptin and obesity-related traits was evaluated in a sample of Brazilian women.

SUBJECTS AND METHODS

Study group

Blood samples were obtained from 228 unrelated Brazilian women, who were selected from Instituto do Coração/Faculdade de Medicina/Universidade de Sao Paulo (FMUSP) and Instituto Dante Pazzanese de Cardiologia (IDPC), Sao Paulo, Brazil. Although these individuals were declared non-Africans by physical evaluation, a lack of relationship between color and genomic ancestry have been found in Brazilian samples (13). The study was previously approved by the FMUSP and IDPC Ethical Committees and written consent was obtained from each participant.

Anthropometrics measurements, such as BMI, waist circumference (WC) and waist-to-hip ratio (WHR) were taken from each participant (14). Systolic/diastolic blood pressure was measured in supine position after resting for 30 min. Based on World Health Organization recommendations, people with BMI higher than 30 kg/m² were classified as obese (15) and those with systolic/diastolic blood pressure over 140/90 mmHg or under lowering-pressure therapy were considered hypertensive (16). The presence of coronary artery disease (CAD) was investigated by coronary angiography.

Biochemical analyses

A 12-hour fasting blood sample was collected from each participant for serum lipids, plasma glucose and leptin determinations. Glucose, triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol were measured by enzymatic-colorimetric assays using a Roche-Hitachi 912 automated analyzer (Hitachi, Nakakojo, Japan). Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol were calculated by Friedewald formulae (17). Plasma leptin was determined by ELISA method (Alexis Biochemical/Vendor BioAgency, Sao Paulo, Brazil). The Atherogenic Index of Plasma (AIP) calculated as log (triglyceride/HDL cholesterol) and the apolipoprotein B/apolipoprotein AI (apoB/ApoAI) ratio were also estimated (18,19).

DNA extraction and *LEP* G-2548A genotyping

Genomic DNA was extracted from 1 mL EDTA-anticoagulated whole blood by a salting-out method (20). G-2548A *LEP* polymorphism was detected by a PCR-RFLP strategy using the endonuclease *Alw*44I (Fermentas, Vilnius, Lithuania). The primers used in PCR assays were designed based on the *LEP* promoter sequence previously described (21). The forward primer (5'-CTTTTGTTTTGTTTTGCGACAGGGG-TGC-3') creates a recognition site for *Alw*44I endonuclease that allows the detection of the A allele. The reverse primer (5'-GCTCCCTTTGCCCGACCC-CG-3') generates a PCR product with a constitutive site for *Alw*44I that is useful as an internal control for restrictive reaction.

Genomic DNA (50 ng) was amplified in 50 µL assays containing primers 200 nmol/L (Life Technologies, Sao Paulo, Brazil), dNTPs 200 µmol/L (Amersham Bioscience, USA), DNA polymerase 1 U and PCR buffer [50 mM KCl, 2 mmol/L $MgCl_2$, 20 mmol/L $(NH_4)_2SO_4$, 75 mmol/L Tris-HCl, pH 9,0] (Biotools, Madrid, Spain).

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PCR assays were carried out in a PTC-200™ Thermal Cycler (M&J Research, Inc., Watertown, USA). After initial denaturation at 96°C for 3 min, amplification was performed using 30 cycles at 96°C for 30 s and 70°C for 90 s, followed by final extension at 72°C for 10 min. PCR products were incubated with 4 U of *Alw44*I (Fermentas, Vilnius, Lithuania) at 37ºC for 2 hs. PCR and restriction products were separated on 2% agarose gel electrophoresis at 100 V for 45 min and analyzed under UV light after ethidium bromide staining.

The accuracy of the genotyping was evaluated by performing duplicate analysis of 20% samples randomly selected. Moreover, a heterozygous *LEP* G-2548A polymorphism sample (control sample) was included in each run.

Statistical analysis

Categorical variables were compared by chi-square test. The agreement of genotypes frequencies with Hardy-Weinberg equilibrium expectations was tested using the chi-square test. Continuous variables were presented as means ± SD and compared by *t*-test. Variables without normal distribution (BMI, WC, leptin, glucose and lipid profile) were log transformed for analysis. Relationships between the genotypes and categorical variables (obesity, diabetes, hypertension, smoking, physical activity and CAD) were evaluated by either the chi-square test or Exact Fisher test.

Logistic Regression Analysis was used to establish correlations between obesity and independent variables. Multivariate Logistic Regression Analysis with Stepwise criteria for variable selection was used to establish correlation with obesity. The relation between *LEP* G-2548A alleles and the dependent variables (BMI, WC and WHR) was evaluated by Univariate Linear Regression Analysis. The results of these analyses were adjusted for the covariates: age, hypertension, diabetes, smoking, physical activity and CAD. The main predictors in the linear model were the *LEP* alleles. Statistical tests were performed by SAS System for Windows software version 8.02 (SAS Institute Inc, 1999-2001, Cary, NC, USA). Significance was assumed for $P < 0.05$.

RESULTS

Anthropometric and clinical data of women are shown in Table 1. Obese individuals exhibited mean age, BMI, waist circumference, waist-hip ratio and plasma leptin values higher than the non-obese group ($p < 0.05$). Hypertension, diabetes, CAD, physical activity and menopause were also more frequent in the obese group ($p < 0.05$). As expected, G-2548A *LEP* genotype distributions did not differ from those expected under Hardy-Weinberg equilibrium (data not shown). There was no difference in genotype and allele frequencies between obese and non-obese groups ($p = 0.304$).

Table 1. Anthropometric, clinical, and *LEP* G-2548A polymorphism data in women.

	Obese (100)	Non-obese (128)	P value
Age (yrs)	52 ± 11	47 ± 13	< 0.001
BMI (kg/m^2)*	34.6 ± 4.1	23.7 ± 2.8	< 0.001
Waist circumference*	106.4 ± 9.2	84.8 ± 10.9	< 0.001
Waist-hip ratio	0.90 ± 0.05	0l.85 ± 0.07	< 0.001
Leptin*	41.9 ± 19.6	15.7 ± 13.1	< 0.001
CAD	14%	1%	0.004
Diabetes	23%	0%	< 0.001
Hypertension	72%	10%	< 0.001
Physical activity	79%	63%	0.032
Menopause	67% (67)	47% (60)	0.01
Smoking	35%	27%	0.340
LEP G-2548A			
Genotype GG	40.0%	30.5%	0.304
Genotype GA	48.0%	53.9%	
Genotype AA	12.0%	15.6%	
G allele	64%	58%	0.133

Number of individuals in parenthesis. Individuals with BMI ≥ 30 kg/m² were classified as obese (15) and those with systolic/diastolic blood pressure over to 140/90 mmHg or under anti-hypertensive therapy were considered hypertensive (16); Continuous variables are presented as mean ± SD and were compared by *t*-test. Categorical variables were compared by qui-square or Exact Fisher test;(*) Values were log transformed.

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Univariate logistic regression analysis showed that women with hypertension and CAD had high risk (eleven times) of obesity (Table 2). Increased age, plasma leptin and glucose, and serum triglycerides, VLDL cholesterol, apoB, apoB/ApoAI ratio and AIP were also associated with obesity in our sample (p < 0.001).

Table 2. Univariate logistic regression analysis of the variables associated with obesity.

Variables	P value	Odds Ratio	95% Confidence Interval
CAD	0.021	11.61	1.45-93.28
Hypertension	< 0.001	24.51	9.63-62.40
Physical activity	0.034	2.23	1.06-4.68
Age	< 0.001	1.04	1.02-1.07
Leptin	< 0.001	1.10	1.07-1.13
Glucose	< 0.001	1.1	1.06-1.14
Total cholesterol	0.066	1.01	0.99-1.01
LDL cholesterol	0.056	1.01	0.99-1.01
HDL cholesterol	< 0.001	0.96	0.94-0.98
VLDL cholesterol	< 0.001	1.08	1.05-1.11
Triglycerides	< 0.001	1.01	1.01-1.02
ApoAI	0.057	0.99	0.98-1.01
ApoB	< 0.001	1.03	1.1-1.04
ApoB/ApoAI ratio	< 0.001	36.69	6.28-214.28
AIP	< 0.001	30.28	9.19-99.74
LEP G allele adjusted*	0.041	4.11	1.06-15.90

(*) Adjusted to the covariates: age, hypertension, CAD, smoking and physical activity.
ApoAI, apolipoprotein I; ApoB, apolipoprotein B; AIP, atherogenic index of the plasma; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

Analyses of *LEP* G-2458A polymorphism showed that women carrying –2548G allele (GG genotype) had four times higher risk of obesity (OR: 4.11, 95% CI: 1.06-15.90) than the –2548A allele carriers (p = 0.041) when the variable was adjusted to the covariates age, hypertension, CAD, smoking and physical activity (Table 2). Univariate linear regression analyses showed that –2548G allele is associated with plasma leptin (R^2: 2.70, p = 0.024) and BMI (R^2: 2.07, p=0.027) but not with waist circumference or waist-hip ratio (Table 3). G allele contributes to the increase in 0.18 log units for leptin and 0.04 log units for BMI.

Table 3. Univariate linear regression analysis of the variables associated with *LEP* G-2548A polymorphism.

Dependent variables	*LEP G*-2548A	P value	R^2 (%)	Estimate (SE)
Body mass index	A allele	0.394	0.31	–0.01 (0.01)
	G allele	0.027	2.07	0.04 (0.02)
Waist circumference	A allele	0.523	0.18	–0.01(0.01)
	G allele	0.167	0.85	0.02 (0.01))
Waist-hip ratio	A allele	0.900	0.01	–0.01 (0.01)
	G allele	0.790	0.04	0.01 (0.01)
Plasma Leptin	A allele	0.876	0.01	0.01 (0.06)
	G allele	0.024	2.74	0.18 (0.08)

Variables were adjusted to the covariates: age, hypertension, diabetes, CAD, smoking and physical activity. Estimate indicates variation of log values.
Results from multivariate logistic regression analysis with Stepwise criterion for variable selection indicate that the risk of obesity was nine times higher in hypertensive women (O.R.: 9.06, 95% CI: 2.67-30.80, p < 0.001) (data not shown). Other predictors for obesity found in our sample are increased plasma leptin (O.R.: 1.08, 95% CI: 1.05-1.12, p < 0.001) and serum apoB (O.R.: 1.03, 95% CI: 1.01-1.05, p < 0.001), and reduced HDL cholesterol (O.R.: 0.93, 95% CI: 0.89-0.97).

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DISCUSSION

The frequency of the *LEP* -2548G allele (64%) observed in Brazilian obese women was similar to that found in European overweight women and obese girls (10,11), as well as in North Americans (22). Our results clearly demonstrate that women carrying –2548G allele have at least four times more risk of obesity than non-carriers suggesting that *LEP* G-2548A polymorphism is a strong obesity predictor.

An association of *LEP* G-2548A polymorphism and increased BMI was also found in overweight Europeans (10) and in a sample of Taiwanese Aborigines with extreme obesity (23). In addition, common variants located in the 5´region of the *LEP*, including G-2548A, were associated with increased BMI in men (24). On the other hand, other studies have failed to demonstrate association among these polymorphisms and obesity or increased BMI (25-28).

The relationship between *LEP* –2548G allele and increased plasma leptin concentrations found in this sample was also described in obese and diabetic individuals from European and Asian populations (11,29). This allele was associated with plasma free leptin levels through an interaction with adiposity and gender in healthy individuals from Greece (30). On the other hand, -2458AA genotype was associated with increased plasma leptin in obese individuals and in men from France cohorts (9,10). These different results may be due to interactions of G-2548A polymorphism with other variants in leptin and/or leptin receptor genes, as well as other variables such as gender, sample size and population, or the model used in genetics analyses.

The G-2548A polymorphism is located at the 5'end of the promoter region of *LEP* and it has been suggested that this remote region may contain inhibitory elements for transcription in adipocytes (21). Even though this polymorphism is close to a SP-1 transcription factor binding site, as well as two repetitive sequences MER11 and Alu that maybe regulate *LEP* transcription, the effect of the G to A substitution at –2548 nucleotide in leptin expression remains to be elucidated.

In this study, obesity was associated with hypertension and other CAD-related risk factors such as advanced age, diabetes, dyslipidemia, smoking, and increased apoB/apoAI ratio and AIP. However, our data do not support association of the *LEP* G-2548A with CAD, as it has been recently demonstrated for the LEP-tet microsatellite polymorphism in Italians and Brazilians (31,32).

Positive correlations between leptin and glucose, lipids, apoB/apoAI ratio and AIP values suggest its possible role as risk factor for CAD. A relationship among leptin, adiponectin, and abdominal obesity with cardiovascular risk assessed by apoB/ApoI ratio was recently demonstrated in Asian Indian and Caucasian populations (33). Leptin exerts many potentially atherogenic effects such as induction of endothelial dysfunction, stimulation of inflammatory reaction, oxidative stress, decrease in paraoxonase activity, platelet aggregation, migration, hypertrophy and proliferation of vascular smooth muscle cells (34). These effects may contribute to the pathogenesis of hypertension, atherosclerosis, and left ventricular hypertrophy frequently associated with obesity (35,36).

In conclusion, *LEP* G-2548A polymorphism is an important predictor for increased plasma leptin and BMI in Brazilian women and it maybe a useful marker for obesity-related risk in this population.

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Endereço para correspondência:

Rosario Dominguez Crespo Hirata
Faculdade de Ciências Farmacêuticas da Universidade de São Paulo
Av. Lineu Prestes, 580, B17
05508-900 São Paulo, SP
E-mail: rosariohirata@usp.br

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