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ORIGINAL ARTICLE

Association of A-604G *ghrelin* gene polymorphism and serum ghrelin levels with the risk of obesity in a mexican population

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Abstract Obesity is a metabolic disorder that has a multifactorial etiology and affects millions of people worldwide. Ghrelin, a hormone coded by the *GHRL* gene, plays a role in human body composition and appetite. Single nucleotide polymorphisms (SNPs) of the *GHRL* gene have been associated with obesity and metabolic disorders. To evaluate the association of A-604G SNP of *GHRL* promoter region with serum ghrelin levels and the risk of obesity in a Mexican population. Two hundred and fifty individuals were enrolled and classified as obese or control subjects (CS) according to BMI. DNA samples, anthropometric measurements and biochemical parameters were obtained from all subjects. The A-604G SNP was genotyped using PCR-RFLPs technique. Ghrelin levels were measured using a commercial enzyme immunoassay. The G/G genotype was more frequent among obese individuals ($p<0.0001$) when compared to CS. The G/A genotype and A allele were associated with protection against obesity (OR 0.29, $p<0.0001$; OR 0.39, $p<0.0001$ respectively), the A allele remained significant after adjusting for age and gender (OR: 0.25, $p<0.0001$). Serum ghrelin levels were higher in obese patients ($p=0.004$) than in CS, however, significance was lost after adjustment for age ($p=0.088$). The G/G genotype was associated with higher levels of serum ghrelin ($p=0.02$) independently of the effect of age. The G/G genotype of the A-604G SNP in the *GHRL* gene is associated with altered serum ghrelin levels and obesity. The A allele was also associated with protection against obesity in this study.

Keywords Obesity · Ghrelin · Polymorphism

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Introduction

Obesity is the most frequent metabolic disorder. It is originated by an imbalance between daily energy consumption and expenditure [1]. It is considered a chronic disease and its major feature is the increase of adipose tissue, which can have important metabolic activity when distributed abdominally (visceral fat) [2]. In the last 30 years obesity has doubled its prevalence worldwide reaching a stunning 13% in 2014, and Mexico is one of the most affected countries worldwide [3]. Obesity and its complications are associated to other diseases such as: type 2 diabetes mellitus (T2D), cancer, stroke, and cardiovascular disease just to name a few [4]. The etiology of obesity is multifactorial. The factors that participate in the development of obesity are: individual's lifestyle, eating behavior, culture, and genetic background [1]. A significant number of genes have been associated with obesity development [5]. The *GHRL* gene, located on chromosome 3p25-26, codes two recently discovered peptides; ghrelin and obestatin [6]. Ghrelin is a 28 amino acid peptide hormone secreted mainly by the

oxyntic cells found in the gastric mucosa. The first reported function was as the endogenous ligand for the growth hormone secretagogue receptor, thus stimulating the release of growth hormone [7]. To date, it is well known that ghrelin increases appetite and food intake through its action in the hypothalamus. Moreover, by acting in other tissues, it decreases thermogenesis, increases cardiac output, gastric emptying, gastric motility, and promotes lipogenesis [7]. Ghrelin serum levels are decreased in obese individuals when compared to lean subjects suggesting a potential role in body weight modulation [8]. The *GHRL* gene has been distinguished as a candidate gene for obesity development in humans [9]. Within its structure, the *GHRL* gene has 12 single nucleotide polymorphisms (SNP), although their functional consequences have not yet been fully elucidated, the A-604G (rs27647) polymorphism in the promoter region has been previously associated with metabolic effects [10, 11]. Nevertheless, its association with ghrelin serum levels and susceptibility to obesity has not been evaluated in the Mexican population. Therefore, the aim of this study is to analyze the association between this SNP with serum levels of ghrelin and the risk of obesity in a Mexican population.

Materials and methods

Study population

Two hundred and fifty adult individuals aged ≥30 years old were enrolled in the present study. They were classified according to their body mass index (BMI) into two groups: obese individuals (BMI ≥ 30 kg/m^2) and control subjects (CS; BMI between 19 and 24.9 kg/m^2). All patients with a history of diabetes mellitus, cancer, rheumatologic, thyroid, parathyroid, kidney and/or liver disease were excluded. The local ethics committee approved this study and all patients provided written informed consent before enrollment in accordance with the Declaration of Helsinki.

Anthropometric measurements

According to the International Society for the Advancement of Kinanthropometry standards; waist and hip circumference (cm), and BMI (kg/m^2), were measured in all subjects. Using a bioelectrical impedance scale (TBF-300A, Tanita®, Tokyo, Japan) we obtained the weight, BMI, and body fat percentage (BFP) from all participants.

Biochemical and hormonal analysis

We determined the concentrations of fasting plasma glucose (FPG), total cholesterol (TC), high-density lipoprotein cholesterol, (HDL-C), and triglycerides (TG) after an overnight fasting period, by means of standard biochemical methods (BioSystems®, Barcelona, Spain). Total plasma ghrelin levels were measured in a subsample of the study population by enzyme immunoassay with a detection range of 0.1–1000 ng/mL (RayBiothech, Inc., Norcross GA, USA).

Genotyping

DNA was isolated from peripheral blood using the Miller method. A-604G genotyping was performed using polymerase chain reaction of restriction fragment-length polymorphisms (PCR-RFLPs). The region of the *GHRL* gene containing the SNP was amplified by PCR using the primers: forward 5′-CACAGCAACAAAGCTGCACC-3′ and reverse 5′-AAGTCCAGCCAGAGCATGCC-3′. PCR was performed in a final volume of 25 µL, containing 200 ng of gDNA, 20 µM of each primer, 1.5 U/µL of Taq polymerase (Fermentas Thermo Fisher Scientific®, Waltham MA, USA), 1× buffer, and 0.1 mM of each dNTP (Dongsheng Biotech Co., Guangdong, China). The amplification was performed on a programmable thermocycler (TC-300, Techne®, Staffs, UK). PCR conditions were: denaturation (94 °C 30 s), annealing (60 °C, 30 s) and extension (72 °C, 30 s) for 36 cycles. Ten microliters of the amplified fragments were incubated with 1.5 U of *DraI* restriction enzyme (New England Biolabs®, Ipswich MA, USA) for 30 min. PCR fragments and digestion products were analyzed in 2% agarose gel stained with Gel Red™ (Biotium, Inc., Hayward CA, USA).

Statistical analysis

Genotypic and allelic distributions were compared using Chi square test. Quantitative variables were compared by means of Student T-test and Mann–Whitney U test, according to their distribution. Odds ratio (OR) was used to evaluate the association between alleles or genotypes and obesity. Multiple and logistic regression models were performed in order to adjust serum ghrelin levels and genotype results. A two-tailed p-value < 0.05 was considered statistically significant. All analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software®, La Jolla CA, USA).

Results and discussion

Clinical and demographical characteristics of all participants are shown in Table 1. As expected, the obese group showed higher values in all anthropometric measures compared to CS (Weight $p < 0.0001$, BMI $p < 0.0001$, Waist

Table 1 Clinical and demographical characteristics of study groups

	Obese n = 125	Controls n = 125	p
Anthropometric			
Gender			
Female n (%)	82 (65.6)	86 (68.8)	NS
Male n (%)	43 (34.4)	39 (31.2)	NS
Age (years)	44.3 ± 7.1	40.3 ± 8.8	0.01
Weight (Kg)	90.6 ± 15.3	63.1 ± 8.7	<0.0001
BMI (Kg/m^2)	34.0 ± 4.0	23.3 ± 1.6	<0.0001
Waist (cm)	102.6 ± 12.0	80.5 ± 7.7	<0.0001
Hip (cm)	113.5 ± 14.1	98.5 ± 4.9	<0.0001
BFP (%)	40.0 ± 6.9	27.1 ± 6.7	<0.0001
Metabolic			
Glucose (mg/dL)	92.4 ± 14.8	89.2 ± 14.5	NS
Total cholesterol (mg/dL)	200.0 ± 40.8	194.2 ± 76.9	NS
Triglycerides (mg/dL)	119.2 ± 77.7	121.9 ± 88.6	NS
HDL-C (mg/dL)	43.0 ± 13.2	50.8 ± 13.7	<0.001
LDL-C (mg/dL)	137.5 ± 40.3	126.1 ± 45.8	NS

Values of gender are expressed as frequency (percentage), Chi-squared test. The rest data are means ± SD, Student's t-test

BMI body mass index, *BFP* body fat percentage, *HDL-C* cholesterol bound to high density lipoprotein, *LDL-C* cholesterol bound to low density lipoprotein, *NS* non significant

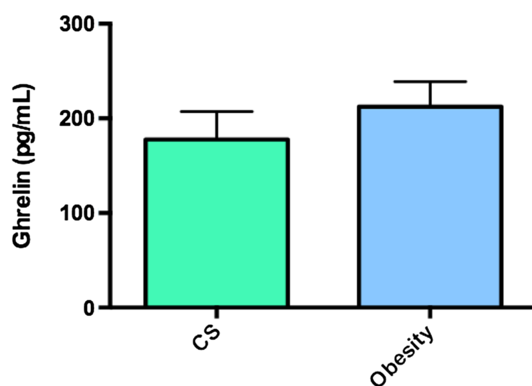

Fig. 1 Ghrelin serum levels among study groups. p = 0.088, *CS* control subjects. Values are expressed as median (interquartile range), multiple regression analysis

circumference p < 0.0001, Hip circumference p < 0.0001 and BFP p < 0.0001). In addition, HDL-C was significantly lower in obese individuals than in CS (43.0 ± 13.2 mg/dL vs. 50.8 ± 13.7 mg/dL, p = 0.001). These results are consistent with the altered lipid profile frequently encountered in the obese population [12].

Ghrelin levels were higher in obese subjects (212.1 (87.2) pg/mL) compared with CS (177.6 (72.8) pg/mL) p = 0.04 (Fig. 1) contrary to what other authors have found [8, 11, 13]. Since previous studies have reported a correlation between ghrelin levels and age [14], we performed an adjusted analysis to exclude the effect of age. After adjustment, significance was lost, although a trend could still be detected (p = 0.088). Regarding this matter previous studies have reported that obese mice have higher serum levels of gastric inhibitory peptide (GIP). It is well known that GIP stimulates the production and secretion of ghrelin. Additionally, high fat diet-induced obesity in mice increases the Rfx6 transcription factor, which is known to increase ghrelin production [15]. In humans, high fat diets (a common feature in the obese population) reduce the ghrelin receptor expression in the hypothalamus, thus generating central ghrelin production [16]. These findings suggest that the reduced ghrelin receptor expression can blunt the signal transmission between stomach and brain generating a compensatory elevation of this hormone [17]. Recent studies show that in high fat diet-induced obese mice, ghrelin producing cells were increased by 15% when compared to control group [18]. Supporting this data, ghrelin expressing cells are increased in obese patients, showing evidence that obesity possibly modifies gastrointestinal cells by inflammatory mechanisms [19]. Moreover, it is important to note that ghrelin is present in the bloodstream in two major forms: desacylated and acylated ghrelin, this last one possesses an octanoic acid that is added by the ghrelin-O-acyltransferase enzyme (GOAT) [20]. Previous studies have shown differential associations between these two different forms of ghrelin. One study [21] reported negative association of total ghrelin and des-acyl ghrelin with BMI and only a positive one with acyl ghrelin. On the opposite, another study [22] showed negative correlation of all forms of ghrelin and BMI. Acyl ghrelin is the form that binds the ghrelin receptor in the brain thus promoting food intake [7] and probably is one of the responsible factors for the increment in BMI. To this respect, it should be noticed that in this study we could only determine total ghrelin levels and did not separately measure the forms of ghrelin.

Genotype distributions of the A-604G SNP are shown in Table 2. Genotypic frequencies were different among study groups (p < 0.0001). The G/G genotype was the most frequent in both groups, but significantly higher in the obese group. Our population genotype frequencies differ from previous studies [23]. The G/A genotype and A allele were associated with a decreased risk of obesity (OR 0.29, IC95% 0.17–0.52, p < 0.0001 and OR 0.39, IC95% 0.25–0.63, p < 0.0001 respectively), after an adjustment by age and gender, G/A genotype was no longer associated with obesity risk (p = 0.90), but the A allele remained as a significant protective factor (OR 0.25, IC95% 0.14–0.45, p < 0.0001), compared to G allele. In other studies and according with our results, the A allele has been previously associated with protection against insulin resistance, while the G allele has been associated with higher BMI [23, 24].

Table 2 GHRL genotypes and allele distribution in obese and CS

Genotype	Frequency n (%)		p	OR	IC95%	p^b
	CS	Obese				
G/G[a]	62 (49.6)	95 (76.0)	$p < 0.0001$	3.22	1.88–5.52	$p < 0.001$
G/A	56 (44.8)	25 (20.0)		0.31	0.22–2.28	NS
A/A	7 (5.6)	5 (4.0)		NS	NS	NS
Allele						
G[a]	180 (72.0)	215 (86.0)	$p < 0.0001$	–	–	–
A	70 (28.0)	33 (13.2)		0.25	0.14–0.45	$p < 0.0001$

NS non significant

[a]Genotype and allele of reference. Genotypes showed Hardy–Weinberg equilibrium; Chi-squared test

[b]Adjusted by age and gender using a multivariate logistic regression analysis

Serum ghrelin levels were significantly different among genotypes. The G/G genotype showed increased ghrelin levels when compared to G/A and A/A genotypes (126 (144.4) pg/mL, 90.0(155.3) pg/mL and 27.7 (44.5) pg/mL respectively, $p = 0.02$) even after adjustment for age. We suggest that the G/G genotype is associated with increased activity of the *GHRL* promoter and a consequent increase in ghrelin serum levels. However, we recognize that the ghrelin serum levels measurements were done only in a small subgroup of the study population, and these results should be interpreted with caution.

It is evident that the ghrelin system has complex regulatory mechanisms that alter its serum levels, these go from dietary factors, changes in gene structure, and post-translational modifications to physiological and pathological conditions [7]. Further studies are required in order to better understand the ghrelin system and its role in metabolic disorders.

Conclusions

The G/G genotype of the A-604G polymorphism is associated with increased levels of serum ghrelin and susceptibility to obesity. The A allele is a protective factor against obesity. Obese individuals tend to have higher serum ghrelin levels when compared to CS in a Mexican population.

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