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Relation of the rs6923761 Gene Variant in Glucagon-Like Peptide 1 Receptor with Weight, Cardiovascular Risk Factor, and Serum Adipokine Levels in Obese Female Subjects

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Background: Studies of the glucagon-like peptide 1 (GLP-1) receptor have been directed at identifying polymorphisms in the GLP-1 receptor gene that may be a contributing factor in the pathogenesis of diabetes mellitus and cardiovascular risk factors. Nevertheless, the role of *GLP-1* variants on body weight, cardiovascular risk factors, and adipokines remains unclear in obese patients. *Objective:* Our aim was to analyze the effects of rs6923761 *GLP-1 receptor* polymorphism on body weight, cardiovascular risk factors, and serum adipokine levels in nondiabetic obese females. *Design:* A sample of 645 obese nondiabetic Caucasian females was enrolled in a prospective way. Basal fasting glucose, c-reactive protein (CRP), insulin, insulin resistance (homeostasis model assessment (HOMA)), total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides concentration, and adipokines were measured. Weights, body mass index (BMI), waist circumference, fat mass by bioimpedance, and blood pressure measures were measured. *Results:* Three hundred and twenty-seven participants (50.7%) had the genotype *GG* and 318 (49.3%) study subjects had the next genotypes; *GA* (270 study subjects, 41.9%) or *AA* (48 study subjects, 7.4%) (second group). In wild group (GG genotype), BMI (1.8 ± 2.3 kg/m^2; $P < 0.05$), weight (3.1 ± 1.3 kg; $P < 0.05$), fat mass (2.4 ± 1.1 kg; $P < 0.05$), waist circumference (2.7 ± 1.9 cm; $P < 0.05$), triglyceride levels (10.4 ± 5.3 mg/dl; $P < 0.05$), interleukin 6 (IL-6) (1.5 ± 0.9 ng/dl; $P < 0.05$), resistin (1.1 ± 0.3 ng/dl; $P < 0.05$), and leptin (30.1 ± 10.3 ng/dl; $P < 0.05$) levels were higher than mutant group (GA + AA). *Conclusion:* Data from our study revealed an association with decreased metabolic and cardiovascular markers in obese females. BMI weight, fat mass, waist circumference, triglycerides, leptin, resistin, and IL-6 serum levels were lower in subjects with A allele than non-A allele subjects. J. Clin. Lab. Anal. 29:100–105, 2015. © 2014 Wiley Periodicals, Inc.

Key words: adipokines; cardiovascular risk factors; glucagon-like peptide 1 receptor; rs6923761; weight

INTRODUCTION

Glucagon-like peptide 1 (GLP-1, 7–36 amide) is an enteroendocrine hormone that plays an important physiological role in maintaining blood glucose homeostasis and dietary intake (1). This hormone is produced by enteroendocrine L-cells primarily localized in the ileal/colonic mucosa. Following food ingestion, GLP-1 is secreted into the blood circulation and acts on multiple target tissues (pancreatic islets, brain, heart, kidney, and gastrointestinal tract) to attenuate the postprandial increase in blood glucose levels, enhances glucose-induced insulin secretion as well as stimulates the growth

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of insulin-producing beta cells, delay of gastric emptying thereby reducing postprandial glucose excursions (2).

GLP-1 acts via a cognate G-protein-coupled receptor (GLP-1R). GLP-1R is expressed predominantly in islets, lung, and brain (3). The receptor is a 463 amino acid long and contains seven transmembrane domains belonging to a family of G-protein coupled receptors (4). Studies of the GLP-1R have been directed at identifying polymorphisms in the GLP-1R gene that may be a contributing factor in the pathogenesis of diabetes mellitus and modulate cardiovascular risk factors. A recent study of individuals with type 2 diabetes mellitus identified a patient with a heterozygous GLP-1R missense polymorphism that resulted in substitution of threonine 149 by methionine (T149M) (rs112198). In contrast to several other GLP-1R polymorphisms that were found in this study (5), the T149M substitution was not observed in a control population of 318 nondiabetic individuals. In other study, Beinborn et al. (6) demonstrated that the T149M substitution, when introduced into the human GLP-1R by recombinant DNA techniques, causes a significant loss of function versus the wild-type protein. In other study (7), two genetic variations in GLP1R (rs6923761 and rs3765467) decreased binding affinity and modestly reduced insulin secretion following GLP-1 infusion in healthy subjects, and the genetic variant of GLP1-R rs6923761 is most frequent of all studied variants.

GLP-1 is also involved in the regulation of food intake and body weight (2). The current view of adipose tissue is that of an active secretor organ, producing and secreting to signals that modulate appetite, insulin sensitivity, energy expenditure, inflammation, and cardiovascular risk factors. It is not well investigated whether GLP-1 might improve insulin resistance and pancreatic beta-cell function, at least in part, by modulating adipokine levels. No studies are reported yet on interaction among rs6923761 variant of *GLP-1 receptor* and adipokine levels have been realized in nondiabetic obese patients. Adipokines are proteins produced mainly by adipocytes (8). These molecules have been shown to be involved in the pathogenesis of the metabolic syndrome and cardiovascular disease, such as adiponectin, leptin, and resistin (9–12).

Taking into account, the high prevalence of this polymorphism and the high rates of obesity in females, we decided to analyze the effects of rs6923761 *GLP-1 receptor* polymorphism on body weight, cardiovascular risk factors, and serum adipokine levels in nondiabetic obese females.

Subjects

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving patients were approved by the HURH ethics committee. All participants provided informed consent to a protocol approved by the local ethical review boards. A sample of 645 obese nondiabetic Caucasian females was enrolled in a prospective way. Inclusion criteria were body mass index (BMI) > 30 kg/m^2 and absence of weight-reducing diet during the 3 months prior to the study.

Exclusion criteria included history of cardiovascular disease or stroke during the previous 24 months, total cholesterol > 200 mg/dl, triglycerides > 250 mg/dl, blood pressure $> 140/90$ mmHg, fasting plasma glucose > 126 mg/dl, as well as the use of sulfonylurea, thiazolidinedions, insulin, glucocorticoids, antineoplastic agents, angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, psychoactive medications, and a hypocaloric diet in previous 12 months.

Procedure

Whole blood samples were taken in the morning after an overnight fast. Basal fasting glucose, c-reactive protein (CRP), insulin, insulin resistance (homeostasis model assessment (HOMA)), total cholesterol, LDL cholesterol, HDL cholesterol, plasma triglycerides concentration, and adipokines (leptin, adiponectin, resistin), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) levels were measured. Weight, height, a tetrapolar bioimpedance, waist circumference, and blood pressure measures were realized. These measures were realized at same time of the day (morning). Genotype of rs6923761 *GLP-1 receptor* gene polymorphism was studied.

All enrolled subjects received instruction to record their daily dietary intake for 3 days including a weekend day. Patients received prospective serial assessment of nutritional intake with 3 days written food records. Handling of the dietary data was by means of a personal computer equipped with personal software, incorporating use of food scales and models to enhance portion size accuracy. Records were reviewed by a dietitian and analyzed with a computer-based data evaluation system. National composition food tables were used as reference (13).

Genotyping of rs6923761 GLP-1 Receptor Gene Polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 5.0 (Premier Biosoft International®, LA, CA). The polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 μL of each oligonucleotide primer (primer forward: 5′-GTTCCTCTACATCATCTACAC-3′ and reverse 5′-CTGCTTCATTCCTCTATCTG-3′) and 0.25 μL of each probes (wild probe: 5′-Fam-CGATCCTCCTCGG CTTCAGGTA-BHQ-1–3′ and mutant probe: 5′-Texas red-CGATCCTCCTCAGCTTCAGGTA-BHQ-2–3′) in a 25 μL final volume (Termociclador iCycler IQ

(Bio-Rad®), Hercules, CA). DNA was denaturated at 95°C for 3 min; this was followed by 45 cycles of denaturation at 95°C for 15 s, and annealing at 58.1°C for 45 s. The PCR was run in a 25 μL final volume containing 12.5 μL of IQTM Supermix (Bio-Rad®) with hot start Taq DNA polymerase. Hardy–Weinberg equilibrium was assessed with a statistical test (chi-square) to compare our expected and observed counts. The two variants were in Hardy–Weinberg equilibrium.

Assays

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyzer 2, Beckman Instruments, Fullerton, CA). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/l (normal range 0.5–30 mUI/l) (14) and the HOMA for insulin resistance were calculated using these values (15). CRP was measured by immunoturbimetry (Roche Diagnostics GmbH, Mannheim, Germany), with a normal range of 0–7 mg/dl and analytical sensitivity 0.5 mg/dl. Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, NY), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate magnesium. LDL cholesterol was calculated using Friedewald formula.

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4–12 ng/ml (16). Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Webster, TX) with a sensitivity of 0.05 ng/ml and a normal range of 10–100 ng/ml (17). Adiponectin was measured by ELISA (R&D systems, Inc., Minneapolis, MN) with a sensitivity of 0.246 ng/ml and a normal range of 8.65–21.43 ng/ml (18). IL-6 and TNF-α were measured by ELISA (R&D systems, Inc.) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL-6 were 1.12–12.5 pg/ml and TNF-α 0.5–15.6 pg/ml (19).

Anthropometric Measurements and Blood Pressure

Body weight was measured to an accuracy of 0.1 kg and BMI computed as body weight/(height^2). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to-hip ratio were measured too. Tetrapolar body electrical bioimpedance was used to determine body composition with an accuracy of 50 g (20). The same investigator measured patients and controls. After introduction of an 800 μA (50 kHz) excitation current, bioimpedance (BIA) measures the geometrical components of electrical impedance Z_e, that is, resistance R (the sum of in-phase vector) and the capacitive component, reactance X (the sum of out-phase vectors) derived from $Z^2 = R^2 + X_c^2$. Precautions taken to ensure valid BIA measurements were no alcohol within 24 h of taking the test, no exercise or food for 4 h before taking the test. Blood pressure was measured twice after a 10 min rest with a random zero mercury sphygmomanometer, and averaged (Omron, LA, CA).

Statistical Analysis

Sample size was calculated to detect differences over 3 kg in body weight loss with 90% power and 5% significance ($n = 300$) in each genotype with a dominant model. The Kolmogorov–Smirnov test was used to determine variable distribution. The results were expressed as average ± standard deviation. Quantitative variables with normal distribution were analyzed with a two-tailed Student's t-test. Nonparametric variables were analyzed with the Mann–Whitney U-test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. A two-way repeated measures ANOVA has been applied for the assessment of the effects of the intervention between both groups. Bonferroni test was used for post-hoc analysis. The statistical analysis was performed for the combined *AA* and *AG* as a group (mutant) and GG genotype as second group (wild), with a dominant model. A P-value < 0.05 was considered significant.

RESULTS

Six hundred and forty-five study obese females gave informed consent and were enrolled in the study. The mean age was 47.5 ± 14.3 years and the mean BMI 36.9 ± 4.2. Three hundred and twenty-seven participants (50.7%) had the genotype *GG* and 318 (49.3%) study subjects had the next genotypes; *GA* (270 study subjects, 41.9%) or *AA* (48 study subjects, 7.4%) (second group). Age was similar in both groups (47.3 ± 15.8 years vs. 47.8 ± 15.0 years: ns).

Table 1 shows the differences in anthropometric variables. In wild group (GG genotype), BMI (1.8 ± 2.3 kg/m^2; $P < 0.05$), weight (3.1 ± 1.3 kg; $P < 0.05$), fat mass (2.4 ± 1.1 kg; $P < 0.05$), and waist circumference (2.7 ± 1.9 cm; $P < 0.05$) were higher than (GA + AA) group. No differences were detected in fat-free mass, waist-to-hip ration, systolic and diastolic blood pressure.

Table 2 shows the differences in cardiovascular risk factors. In wild group, triglyceride levels were higher than mutant group (10.4 ± 5.3 mg/dl; $P < 0.05$). No differences were detected in glucose, total cholesterol,

TABLE 1. Anthropometric Variables in Both Groups of Obese Females

Parameters	*GG* ($n = 327$)	*GA* or AA ($n = 318$)
BMI	37.2 ± 6.8	35.4 ± 6.1*
Weight (kg)	92.7 ± 16.2	89.7 ± 16.8*
Fat-free mass (kg)	48.2 ± 7.8	47.9 ± 7.6
Fat mass (kg)	43.8 ± 12.5	41.3 ± 12.3*
WC (cm)	110.7 ± 14.2	107.6 ± 14.5*
Waist to hip ratio	0.90 ± 0.1	0.89 ± 0.09
Systolic BP (mmHg)	127.3 ± 14.1	127.4 ± 18.5
Diastolic BP (mmHg)	82.3 ± 8.9	80.8 ± 13.4

BMI, body mass index; BP, blood pressure; WC, waist circumference.
*$P < 0.05$.

TABLE 2. Classical Cardiovascular Risk Factors in Both Groups of Obese Females

Parameters	*GG* ($n = 327$)	*GA* or AA ($n = 318$)
Glucose (mg/dl)	100.4 ± 16.2	100.6 ± 20.3
Total ch. (mg/dl)	207.8 ± 32.1	206.3 ± 40.3
LDL-ch. (mg/dl)	128.1 ± 33.9	130.5 ± 36.2
HDL-ch. (mg/dl)	55.5 ± 12.1	56.5 ± 18.9
TG (mg/dl)	122.6 ± 55.8	112.5 ± 50.9*
Insulin (mUI/L)	13.4 ± 8.4	13.3 ± 6.9
HOMA	3.35 ± 2.3	3.37 ± 1.9
CRP (mg/dl)	6.1 ± 6.3	6.7 ± 6.1

Ch., cholesterol; TG, triglycerides; CRP, c-reactive protein; HOMA, homeostasis model assessment.
*$P < 0.05$.

TABLE 3. Dietary Intake in Both Groups of Obese Females

Parameters	*GG* ($n = 327$)	*GA* or AA ($n = 318$)
Energy (kcal/day)	1853.7 ± 732	1756 ± 525
CH (g/day)	197.7 ± 81.1	185.8 ± 66.2
Fat (g/day)	78.8 ± 36.1	75.7 ± 36.2
S-fat (g/day)	22.8 ± 11.1	21.9 ± 10.5
M-fat (g/day)	36.4 ± 13.3	35.2 ± 14.5
P-fat (g/day)	7.5 ± 3.4	7.4 ± 3.8
Protein (g/day)	85.4 ± 25.5	83.3 ± 22.8
Exercise (h/week)	1.73 ± 2.2	1.69 ± 2.1
Dietary fiber	15.17 ± 6.2	15.18 ± 6.5

CH, carbohydrate; S-fat, saturated fat; M-fat, monounsaturated fat; P-fat, polyunsaturated fat. No statistical differences between groups.

LDL cholesterol, HDL cholesterol, insulin, HOMA, and CRP.

Table 3 shows nutritional intake with 3 days written food records. No statistical differences were detected in calorie, carbohydrate, fat, and protein intakes. Distribution of type of fats was similar in both genotypes.

Table 4 shows the differences in adipokine levels. In wild group, IL-6 (1.5 ± 0.9 ng/dl; $P < 0.05$), resistin (1.1 ± 0.3 ng/dl; $P < 0.05$), and leptin (30.1 ± 10.3 ng/dl; $P < 0.05$) levels were higher than mutant group. No differences were detected in TNF-α and adiponectin levels.

TABLE 4. Circulating Adypocitokines in Both Groups of Obese Females

Parameters	*GG* ($n = 327$)	*GA* or AA ($n = 318$)
IL-6 (pg/ml)	3.29 ± 2.1	1.71 ± 2.4*
TNF-α (pg/ml)	9.51 ± 9.1	10.81 ± 4.1
Adiponectin (ng/ml)	24.36 ± 28.8	23.58 ± 29.4
Resistin (ng/ml)	5.91 ± 5.8	4.88 ± 3.8*
Leptin (ng/ml)	71.1 ± 33.1	42.6 ± 38.1*

IL-6, interleukin 6.
*$P < 0.05$.

DISCUSSION

Our cross-sectional study showed an association between the rs6923761 *GLP-1 receptor* polymorphism and body weight, triglycerides, leptin, resistin, and IL-6 serum levels in obese females.

The GLP-1R is a family B peptide hormone G-protein-coupled receptor primarily expressed in pancreatic beta cells that responds to full-length GLP1 and other truncated circulating forms of GLP-1. The presence of single nucleotide polymorphisms (single nucleotide polymorphism (SNPs)) may be linked to the rate onset of cardiovascular risk factors in populations as obese patients. On the other hand, GLP-1R way is one of the new ways to target in the development of treatments to diabetes mellitus type 2. Several GLP-1R polymorphisms have been assessed previously in vitro, although not explored in a wide range of nondiabetic patients (6, 21).

Some authors (22) have identified major pharmacological differences in the signaling profile or allosteric modulation of multiple GLP1R polymorphism, the most detrimental substitution is Thr 149 Met, although the most frequent substitution is Gly 168 Ser (G ->A) rs6923761. The role of this polymorphism has not been studied in a large population of obese female patients, only small studies have been realized (7). In this study, Sathananthan et al. (7) showed an altered insulin secretory response to infused GLP-1 in 88 healthy individuals. Nevertheless, sex distribution or BMI were not reported in the study, only the absence of diabetes was assessed. In contrast, to this small study with a low prevalence of the mutant allele, 29% (7), in our study comprising 645 people shows a prevalence of A allele of 49.3%. At present, this variation in GLP-1R has not been associated with type 2 diabetes mellitus (23).

Results from our study showed a relationship of metabolic parameters with the mutant allele (A) of rs6923761 *GLP-1 receptor* polymorphism in obese females. Triglycerides, leptin, resistin, and IL-6 serum levels

were lower in subjects with A allele than non-A allele carriers. On the other hand, the presence of A allele was associated with lower BMI, weight, fat mass, and waist circumference than the absence of this mutant allele. This is the first study showing such an association in nondiabetic obese people. Tokuyama et al. (5) studied nine types of polymorphisms in GLP-1R and there were no significant differences in BMI and insulin resistance. However, rs6923761 was not evaluated in this study and all evaluated SNPs of GLP-1R had a low frequency than rs6923761. Data from our study showed that obese females with A allele had a lower body weight and fat mass with a low triglyceride levels, low inflammatory markers (IL-6), and low levels of adipokines related with fat mass (resistin and leptin). The mechanism by which GLP-1R variants influence obesity and metabolic profile is unknown. Perhaps, the loss of effect of GLP-1 receptor interacts with adipogenesis and could explain these unclear results. It is also possible that this SNP variant may be capturing functional effects of a flanking linked SNP, and variation of this locus may influence adipogenesis and cardiovascular risk factors. The results of our study suggest that there is no association of GLP-1R with either energy intake or macronutrient composition. Other hypothesis to explain this unclear association could be realized. For example, unknown effects of GLP1-R variants have been showed by Sheikh et al. (24). In this study (24), the polymorphism (leu260Phe) of GLP-1R was associated with morning cortisol in preschoolers with new implications in the endocrine system of GLP1 receptor.

Although, the mechanistic basis for the loss of effect of GLP-1 effect in patients with SNPs in this receptor is unclear. This effect is likely (22) to be chemotype-dependent, when glycine at position 168 is replaced by a serine. Perhaps the GLP-1R signaling pathway is partially disrupted, insulin sensitivity, insulin secretion, and glucose effectiveness would be repaired. Is it impossible to generate a hypothesis on the mechanistic consequences when glycine at position 168 is replaced by a serine. The direction of the effect for rs6923761 in our study is opposite to what would be expected due to the correlation. Further studies are needed to evaluate the molecular basis of our data. In conclusion, our data showed a relationship of metabolic parameters with the mutant allele (A) of rs6923761 *GLP-1 receptor* polymorphism in obese females. BMI weight, fat mass, waist circumference, triglycerides, leptin, resistin, and IL-6 serum levels were lower in subjects with A alelle than non-A allele subjects.

ABBREVIATIONS

BMI = body mass index
CRP = c-reactive protein
GLP-1R = glucagon-like peptide 1 receptor
HOMA = homeostasis model assessment
IL = interleukin
TNF = tumor necrosis factor

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