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ORIGINAL ARTICLE

DUAL EFFECT OF THE *GHRL* GENE VARIANT IN THE MOLECULAR PATHOGENESIS OF OBESITY

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ABSTRACT

Obesity is as a global health problem due to its interaction with complex chronic disorders such as cardiovascular disorders, type 2 diabetes mellitus (T2DM) and cancer. Despite the fact that pathogenesis of obesity is not yet clearly understood, it is associated with a combination of psychological, environmental and various genetic factors. Here, employing a case-control design, we aimed to examine the effects of the *GHRL* c.152C>T (p.Arg51Gln) (rs34911341) and c.214G>T (p.Leu72Met) (rs696217) markers on susceptibility to obesity in a Turkish-Cypriot population, as well as to evaluate whether these markers affect biochemical parameters and show their putative functional consequences. This study involved 211 Turkish-Cypriot subjects (106 obese and 95 non obese). Genotyping for the *GHRL* gene polymorphisms was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Our results indicate that the *GHRL* Leu72Met polymorphism was found to be significantly higher in obese patients, with respect to genotypic ($p = 0.0012$) and allelic ($p = 0.0005$) frequencies. Strikingly, the rs696217 GT genotype (heterozygous) had significantly lower serum high-density lipoprotein cholesterol (HDL-C) ($p = 0.015$) than GG (wild type) genotypes. Overall, Leu72Met susceptibility variant may be considered as risk and crucial marker for both obesity and cholesterol metabolism in the community of Turkish-Cypriots. Thus, the dual effect of the *GHRL* gene Leu72Met variant may be used for clinical diagnosis.

Keywords: Arg51Gln; Ghrelin; *GHRL* gene; Leu72 Met; Obesity; Turkish-Cypriot population.

INTRODUCTION

A worldwide public health problem, obesity shows a complex etiology due to its association with type 2 diabetes mellitus (T2DM), hypertension, cardiovascular disorders as well as cancer. Human adiposity is the result of the complex interaction of mainly social, psychological, environmental and more importantly genetic factors [1]. Obesity is a result of excess weight due to storing of extra calories as fat when a person consumes more calories than they use as energy [2].

Epidemiological studies have shown that glucose intolerance, hypertension and abdominal obesity lead to coronary health diseases [3,4]. Genetic modifiers together with environmental factors have an important role in the susceptibility of obesity [5-7]. Thus, obesity phenotypes that exhibit multifactorial genetic characteristics vary, depending on lifestyle, dietary habits and genetic background of the individual.

Therefore, phenotype-obesity relationship to show which individual is at-risk for developing obesity and which one is resistant to treatment interventions or diet, should be well determined [4-9]. Nevertheless, to determine the interaction between obesity and genetic markers, genetic linkage and association studies were performed to identify the candidate genes causing obesity. In 2005, the Human Obesity Gene Map project conducted 1100 scientific studies on 500 genes, genetic determinants and chromosomal regions that could possibly be associated with human obesity phenotypes, and were reported in particular cohorts [10]. Moreover, to date, approximately

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100 genes have been associated with obesity and coronary heart diseases [11]. Although the results vary according to the evaluated population, genetic variants play a significant role in the development of obesity.

Leptin and ghrelin are proteins that regulate appetite and energy balance in the human body. Previous studies have shown that genetic changes in those genes that encode peptides or receptors are associated with body weight and metabolic abnormalities [12]. Ghrelin, a potent growth hormone stimulator (secretogogue), works in opposition to leptin. It stimulates appetite and nutrition are called orexigenic. Fasting increases the ghrelin level and it drops after 60-120 min. after nutrient intake [13]. Previous studies indicated that, the highest ghrelin level was found in anorexia nervosa and Prader-Willi syndrome [14]. The human ghrelin hormone is composed of 28 amino acid peptides produced from 117 amino acid preproghrelin [15]. The growth hormone secreting receptor (GHSR)'s endogenous ligand, ghrelin, stimulates the appearance of growth hormone (GH). Thus, it plays an important role in apetitis control, body weight and insulin and glucose metabolism [16,17]. Plasma ghrelin levels are inversely proportional to obesity, T2DM as well as insulin resistance [18-20]. Variations within the *GHRL* gene could possibly raise ghrelin protein defects and affect its function.

The *GHRL* gene variants, Leu72Met and Arg51Gln, resulting from nucleotide substitutions on the second exon, have previously been associated with obesity in many populations. The *GHRL* c.214G>T (p.Leu72Met) (rs696217) polymorphism was localized in exon 2 within the *GHRL* gene. The association between the Leu72Met variant and obesity has been shown in obese Italian children and in middle-aged overweight Japanese men [21,22]. On the other hand, no association was found in the Danish population [23]. The *GHRL* Leu72Met variant and obesity-related phenotype and metabolic diseases vary according to different populations. Surprisingly, there is no study to show any association of the *GHRL* c.152C>T (p.Arg51 Gln) (rs34911341) variant and being obese in the literature [24]. Detection of gene variants and modifiers that might regulate the gene expression are crucial. Together with determination of the allele frequencies within the population are significant to precision medicine.

Recently, we have investigated the association between the Adiponectin *(ADIPOQ)*, the fat mass and obesity-associated *(FTO)* and the angiotensin I-converting *(ACE)* genes variants and obesity [25]. There is no investigative study showing the relationship between the *GHRL* gene polymorphisms and obesity in North Cyprus. International databases such as the Human Genome Variation and the American National Library of Medicine have identified gene polymorphisms found in many societies and explained their observed frequencies in the community. Unfortunately, Cyprus and many middle eastern countries such as Turkey do not exist in those databases. In our study, we aimed to investigate the association between the *GHRL* gene variations and obesity in the population of North Cyprus.

MATERIALS AND METHODS

Study Design and Studied Subjects. The current investigation involved 106 adult obese patients and 95 non obese subjects from the Turkish-Cypriot population. Turkish-Cypriots are defined as residing in North Cyprus as well as being born to parents who were born before 1974. Considering the Turkish-Cypriot population in the Island, the sample size is convenient. Every participant was provided with a questionnaire that included ethnicity, age, socioeconomic status and general health conditions. Subjects with systemic and metabolic disorders such as diabetes mellitus T2DM, hypertension, dyslipidemias, cirrhosis, cancer, kidney lithiasis, thyroid, cardiovascular disorders, or any active inflammatory disease, were excluded. The participants did not receive any medications or conduct any dietary or exercise program during the sample collection. Written informed consent was obtained from all the subjects. The study was approved by the Research Ethics Committee of the University [ethics committee application number: YDU/2017/43-354; project no: SAG-2016-2-036].

Anthropometric Measurements and Biochemical Parameters. Anthropometric measurements [height (m), weight (kg), waist circumference (cm) and hip circumference (cm)] were performed at the fasting state from each subject. Hip circumference was measured by placing a measuring tape around fullest portion of the patient's hips. Waist circumference was measured using soft tape. Waist was defined midway between the lowest rib (laterally) and the iliocristale landmark. Body mass index (BMI) was calculated by dividing body weight (kg) by the square of height (m^2). A BMI of >30 kg/m^2 was considered to be obese [26].

Peripheral blood samples were collected after overnight fasting. Serum levels of triglycerides (TG), glucose, high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) were measured using an automated analyzer (Abbott Architect C8000; Abbott Laboratories, Abbott Park, IL, USA). Insulin concentrations were measured using an electrochemiluminescence assay (Ref. 12017547; Elecsys Corporation, Lenexa, KS, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: fasting insulin (μU/mL) × fasting glucose (mmol/L) divided by 22 [27].

Genotyping. Each participant's genomic DNA was isolated from EDTA-containing whole blood using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To minimize the risk of DNA contamination, all procedures were conducted in a class II laminar flow. The genotyping of the *GHRL* rs34911341 (C>T) (Arg51Gln) and rs696217 (G>T) (Leu72Met) was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The detail of PCR primers, restriction endonucleases and digestion patterns of DNA fragments are shown in Table 1 [28]. The PCR reaction was performed on a conventional thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 25 µL containing PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, CA, USA), 10 nM of each primer (Hibrigen Biyoteknoloji Ar-Ge San Tic Ltd. Sti, Gebze, Koceli, Turkey), and ~10 ng of the genomic DNA template. Digested products were segregated on 2.0% agarose gels and visualized by ethidium bromide staining and subsequent UV transillumination. Genotypes were determined on the basis of the presence or absence of restriction sites (Table 1).

Statistical Analyses. Continuous variables were expressed as means ± SD. Comparison between groups were analyzed using Student's *t*-test for continuous variables. Comparison of anthropometric and biochemical parameters between genotypes was performed by the one-way analysis of variance (ANOVA) test. Allele frequencies were calculated and the Hardy-Weinberg equilibrium (HWE) was evaluated by the goodness-of-fit χ^2 test. The odds ratio (OR) and the 95% confidence interval (95% CI) were determined to calculate the strength of the association between genotypic alleles and the obese and non obese subjects. All statistical analyses were carried out using the GraphPad Prism 7 software (GraphPad Software, Inc, San Diego, CA, USA).

RESULTS

Demographic Statistics and Biochemical Parameters. The demographic characteristics and biochemical parameters of the studied individuals are shown in Table 2. This case-control study comprised 211 Turkish-Cypriots (106 obese and 95 non obese). One hundred and six obese adult patients with an age of 41.56 ± 9.87 and BMI of 41.58 ± 4.93 kg/m^2 were a generated case group. The control group included 95 non obese subjects with the mean age of 39.03 ± 9.45 years and BMI mean was 22.61 ± 1.82 kg/m^2. There was no statistical difference of gender between the two groups. The statistically significant difference between biochemical parameters including fasting glucose, TC, LDL-C, HDL-C, TG and HOMA-IR was observed

Table 1. Details of polymerase chain reaction primers and restriction endonucleases used, and of the resulting restriction fragments.

GHRL Polymorphism	PCR Primers (5'>3')	Restriction Enzyme	Digestion Pattern	Ref.
rs34911341 (Arg51Gln)	F: TCC AGC CTG CCA CTT AGC R: GGA CCC TGT TC ACT GCC AC	*Sac*I	C allele: 373 bp; T allele: 210 and 163 bp	[28]
rs696217 (Leu72Met)	F: TCC AGC CTG CCA CTT AGC R: GGA CCC TGT TC ACT GCC AC	*Bsr*I	G allele: 373 bp; T allele: 271 and 101 bp	[28]

PCR: polymerase chain reaction; F: forward; R: reverse.

Table 2. Baseline characteristics of the studied population.

Parameters	Obese Subjects (*n*=106)	Non Obese Subjects (*n*=95)	*p* Value
Age (years)	41.56±9.87	39.03±9.45	0.066
BMI (kg/m^2)	41.58±4.93	22.61±1.82	<0.0001
Waist circumference (cm)	112.40±13.13	85.50±7.33	<0.0001
Hip circumference (cm)	120.50±12.57	99.10±6.14	<0.0001
Fasting glucose (mg/dL)	102.5±23.46	89.60±7.35	<0.0001
Total cholesterol (mg/dL)	231.30±38.17	203.90±35.57	<0.0001
LDL-C (mg/dL)	144.30±33.42	125.70±30.29	<0.0001
HDL-C (mg/dL)	47.40±10.55	54.80±9.36	<0.0001
Triglycerides (mg/dL)	163.10±85.11	102.20±41.22	<0.0001
HOMA-IR	4.60±4.01	2.20±1.13	<0.0001

BMI: body mass index; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostatis model assessment of insulin resistance.

Table 3. The genotype distribution and the allele frequencies of the *GHRL* gene variants. [Results are presented as *n* (%)]

A) Genotype	**Obese Subjects (*n*=106)**	**Non Obese Subjects (*n*=95)**	***p* Value**
rs34911341 (C>T) (Arg51Gln)			
CC	104 (98.12)	95 (100.00)	0.40
CT	1 (0.94)	0 (0.00)	
TT	1 (0.94)	0 (0.00)	
rs696217 (G>T) (Leu72Met)			
GG	37 (34.91)	57 (60.00)	0.0012
GT	57 (53.77)	34 (35.79)	
TT	12 (11.32)	4 (4.21)	
B) Allele Frequency	**Obese Subjects (*n*=106)**	**Non Obese Subjects (*n*=95)**	***p* Value**
rs34911341 (C>T) (Arg51Gln)			
C	209 (98.58)	190 (100.00)	0.25
T	3 (1.42)	0 (0.00)	
rs696217 (G>T) (Leu72Met)			
G	131 (61.79)	148 (77.79)	0.0005
T	81 (38.21)	42 (22.11)	

between obese and non obese (p <0.001 in all parameters) as well as physical parameters of BMI, waist circumferences, hip circumferences showed significant difference (p <0.001 in all parameters) except age (p = 0.066) Table 2).

Genotype Distribution and Allele Frequencies of the *GHRL* Gene Variants (Arg51Gln and Leu72Met) in the Studied Population. The allele frequencies and genotype distributions of the *GHRL* rs34911341 (C>T) (Arg51 Gln) and rs696217 (G>T) (Leu72Met) gene variants are shown in Table 3. Distribution of the *GHRL* rs34911341 (C>T) genotypes were 98.12% for CC, 0.94% for CT and 0.94% for TT in the obese group. The frequencies of CC, CT and TT genotypes were 95, 0.0 and 0.0%, respectively, in non obese subjects. There was significant deviation of genotypic distribution from HWE in obese subjects (χ^2 = 46.43, p <0.05). The deficit of the CT genotype frequencies in obese subjects probably accounts for the deviation from HWE. No significant difference in genotype frequencies of the rs34911341 polymorphism was detected between obese and non obese subjects (p = 0.40).

On the other hand, the rs696217 (G>T) genotype frequencies were calculated and are shown in Table 3. In obese subjects, the genotype frequencies were 34.91% for GG, 53.77% for GT and 11.32% for TT. The frequencies of GG, GT and TT genotypes were 60, 35.79 and 4.12%, respectively, in non obese subjects. There were no significant deviation of genotypic distribution from HWE in both obese (χ^2 = 2.04, p = 0.15) and non obese subjects (χ^2 = 0.14, p = 0.7). A significant difference in genotype frequencies of the rs696217 polymorphism was detected between obese and non obese subjects (p = 0.0012). However, the results were in agreement with the global minor allele frequency (MAF) observations by Ensembl Genome Browser [29]. The MAF of *GHRL* rs696217 T allele was determined as ~38.0% in obese patients and ~22.0% in non obese individuals. The case-control genetic association analysis indicated a statistically significant difference in the allele frequencies of the *GHRL* rs696217 (G>T) variant between obese and non obese subjects [p = 0.0005; odds ratio (OR) = 0.459; 95% confidence interval (95% CI) = 0.295-0.713] (Table 3).

Associations Between the *GHRL* Leu72Met Gene Polymorphisms and Clinical Parameters. The genotype distributions of all studied subjects were tested according to anthropometric and metabolic characteristics. The *GHRL* rs696217 T allele that substituted Leu→Met at position 72 was found to be significant when associated with waist circumference and hip circumference levels in the population (p = 0.005, p = 0.002, respectively) (Table 4). No other statistically significant parameter was observed in other clinical characteristics except for HDL-C (p = 0.018). Strikingly, subjects with the rs696217 GT genotype (heterozygous) had significantly lower HDL-C (p = 0.015) than GG (wild type) subjects (Table 4).

DISCUSSION

The current investigation aimed to evaluate the association between putative obesity-associated *GHRL* rs34911341 (C>T) (Arg51Gln) and rs696217 (G>T) (Leu 72Met) gene markers and their likely effect on obesity pathogenesis in the society of Turkish-Cypriots. To the

Table 4. Anthropometric and metabolic characteristics of all subjects in the genotypes of the rs696217 (G>T) polymorphism. [Data are expressed as mean±SD. Analysis of variance (ANOVA) was performed for comparison of the subgroups; bold *p* values are significant.]

Parameters	GG (*n*=94)	GT (*n*=91)	TT (*n*=16)	*p* Value
Age (years)	40.17±8.48	40.30±11.13	41.88±8.43	0.8
BMI (kg/m^2)	34.00±24.48	31.30±8.68	33.13±9.44	0.81
Waist circumference (cm)	95.61±15.70[a]	102.90±17.43	105.50±20.83	**0.005**
Hip circumference (cm)	106.70±13.87[b,c]	113.10±14.08	116.60±17.23	**0.002**
Fasting glucose (mg/dL)	93.83±12.75	97.62±17.88	105.20±41.12	0.06
Total cholesterol (mg/dL)	221.00±34.66	212.90±43.36	233.40±44.09	0.104
LDL-C (mg/dL)	134.5.0±29.26	134.30±35.43	148.50±41.23	0.266
HDL-C (mg/dL)	52.94±10.61	48.62±10.56[d]	52.25±9.16	**0.018**
Triglycerides (mg/dL)	129.00±64.40	137.80±80.77	145.80±92.20	0.59
HOMA-IR	3502.00±2.20	3246.00±2.40	4522.00±3.90	0.34

BMI: body mass index; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostatis model assessment of insulin resistance.

[a] Significant difference between GG genotype and GT genotype by post-hoc Tukey test ($p = 0.0104$).

[b] Significant difference between GG genotype and GT genotype by post-hoc Tukey test ($p = 0.0068$).

[c] Significant difference between GG genotype and TT genotype by post-hoc Tukey test ($p = 0.028$).

[d] Significant difference between GG genotype and GT genotype by post-hoc Tukey test ($p = 0.015$).

best of our knowledge, this is the first study to evaluate the *GHRL* gene variations in this population and other ethnic populations in the Middle Eastern geographic region. Recent studies have indicated that association studies vary among populations. Since 1974, Turkish-Cypriots have been located in Northern Cyprus having a *de jure* population of only 256,644 [30]. Obesity is a world health problem, and the number of individuals who suffer from obesity also rise day by day in Cyprus. Previously, many gene loci were associated with obesity in different studies [9,10,31]. However, the predisposition effect of these gene loci show differences according to the interaction with environment as well as ethnic origin. Recently, we investigated the relationship between *FTO* rs9939609 (A>T), *ADIPOQ* rs2241766 (G>T), and *ACE* rs4340288 polymorphism and obesity in the current population [25] and our results indicated that the *FTO* gene rs9939609 A allele was found to have a strong association with pathogenesis obesity in Turkish-Cypriots. Additionally, Becer *et al.* [32] did not find direct association between obesity and the *LEPR* gene Q223R polymorphism in Turkish-Cypriots]. Nevertheless, the association between the *GHRL* gene variants and obesity has been a matter of interest in recent years, especially after bariatric surgery becoming a lifesaving trend.

Ghrelin, an orexigenic hormone in humans, is secreted mainly by the stomach stimulating growth hormone release, appetite and food intake, and plays a key role in regulating the energy homeostasis of the organism [32]. Previously, ghrelin polymorphisms were linked to BMI-related obesity and metabolic syndrome with variable results [30,33,34]. As expected, in our study the *GHRL* rs696217 T allele showed a statistically significant association with waist circumference and hip circumference level in all subjects. The *GHRL* Arg51Gln [rs34911341 (C>T)] was associated with hypertension in Caucasian subjects [35], on the other hand, the 51Gln marker has been found to have a protective factor against the development of T2DM [24,36]. The Arg51 residue is a site for proteolytic cleavage to synthesis ghrelin, and its substitution to the 51Gln variant leads to lower ghrelin plasma levels, resulting in less abundant synthesizing of the preproghrelin peptide [37]. In the current study, MAF of the *GHRL* 51Gln allele was less than 0.1% in both obese and non obese groups (0.94 and 0.00%, respectively), which is closely comparable to that reported among the global population (0.003) [29]. Our results failed to show a significant over- or under-representation of any of the *GHRL* Arg51Gln polymorphism in the obese group.

On the other hand, the *GHRL* gene Leu72Met [rs696217 (G>T)] missense variant has been associated with alcohol use disorder, alcohol consumption [38] and bulimina nervosa [39]. Moreover, Steinle *et al.* [40] reported that Met72 allele was associated with metabolic syndrome as well as higher fasting glucose, LDL-C and higher triglyceride levels in the Old Order Amish population. Recent meta-analysis study by Huang *et al.* [41] reported that the *GHRL* gene Leu72Met variation was possibly protective against T2DM in Caucasians and predisposing in Asians. The consequence of c.214G>T

polymorphism is the amino acid substitution Leu72Met. This amino acid residue is outside of the mature ghrelin and the functional results of the nucleotide change is not known [39]. In our study, the frequency of the minor allele rs696217 T (Met72) was found to be significantly higher in obese subjects ($p = 0.0005$). Thus, the *GHRL* rs696217 T single nucleotide polymorphism may be associated with obesity in the population of Turkish-Cypriots.

Subjects with the rs696217 GT genotype (heterozygous) had significantly lower serum HDL-C than GG (wild type) subjects. Su *et al.* [42] showed that carriers of the Met72 allele had significantly lower TG/HDL-C than Leu72 genotype carriers after a high-carbohydrate diet. Thus, these results may suggest that it is not the preproghrelin Leu72Met polymorphism alone that is involved; other potential intervening factors, such as nutritional factors, may also affect plasma lipids profiles.

As well as other genetic epidemiology studies, the limitation of this study investigation was that the number of subjects included in the current study was relatively small, and this might lower the sensitivity. Secondly, due to financial issues we were unable to include serum ghrelin level in the biochemical parameters. Therefore, this study lacked comparisons of the *GHRL* gene markers (Arg51Gln and Leu72Met) and ghrelin hormone.

Conclusions. Overall, the results from this study determine the very good evidence of the homozygous Met72 Met genotype at the in exonic locus on the *GHRL* gene may be an inherited risk factor for developing the obesity pathology in the Turkish-Cypriot population. Along with additional, the *GHRL* gene Leu72Met variant may be offered as a screening option to the patients who come in for obesity clinic as well as medical check-up. Depending on the results, the individuals may be guided to change their lifestyle such as engaging in more physical activities and designing new diet plans.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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