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The Leu72Met (rs696217 G>T) Polymorphism of the Ghrelin Gene Might Be a Protective Factor for Nonalcoholic Fatty Liver Disease

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ABSTRACT

Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is a growing problem and the commonest cause of chronic liver disease throughout the world. Given the strong association between NAFLD and insulin resistance and obesity, as well as the central role of ghrelin in these metabolic disorders, we explored whether ghrelin (*GHRL*) and ghrelin receptor (*GHSR*) gene polymorphisms were associated with susceptibility to NAFLD.
Methods: In this case-control retrospective study which was conducted between April 2010 and July 2013, *GHRL* (rs696217 or Leu72Met) and *GHSR* (rs2922126) gene polymorphisms were genotyped in 153 cases with biopsy-proven NAFLD and 157 controls using the polymerase chain reaction - restriction fragment length polymorphism method.
Results: The *GHRL* rs696217 "GT+TT" genotype or "GT" genotype compared with the "GG" genotype occurred less frequently in the patients with NAFLD than the controls and the differences remained significant after adjustment for confounding factors such as age and body mass index (p=0.018; OR=0.35, 95%CI: 0.14–0.84 and p=0.046; OR=0.40, 95%CI: 0.16–0.98, respectively). Furthermore, the *GHRL* rs696217 'T' allele compared with 'G' allele was significantly underrepresented in the cases (p=0.007; OR=0.33, 95%CI: 0.15-0.76). Nevertheless, no significant difference was found for *GHSR* rs2922126 gene polymorphism.
Conclusions: Our findings suggested, for the first time, that the GHRL rs696217 or Leu72Met "GT+TT" genotype and "GT" genotype compared with "GG" genotype, as well as the "T" or Met72 allele compared with "G" or Leu72 allele had a protective effect for NAFLD susceptibility. However, other studies are required to confirm these findings.

Key words: ghrelin – *GHRL* gene – *GHSR* gene – NAFLD – polymorphism.

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CI: confidence interval; DBP: diastolic blood pressure; GGT: gamma glutamyl transferase; GHRL: ghrelin; GHSR: growth hormone secretagogue receptor; IR: insulin resistance; MAFLD: metabolic dysfunction-associated fatty liver disease; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; OR: odds ratio; PCR-RFLP: polymerase chain reaction - restriction fragment length polymorphism; SBP: systolic blood pressure; SNP: single nucleotide polymorphism; T2D: type 2 diabetes.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), currently known as metabolic dysfunction-associated fatty liver disease (MAFLD) [1, 2], is an emerging global chronic liver disease characterized by accumulation of triglycerides in hepatocytes without excessive alcohol consumption. NAFLD encompasses a broad spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma. The prevalence of NAFLD is very high (25%) and increasing at a rapid rate all over the world [3], but its etiology has not yet been completely clarified. Previous studies, however, have reported that insulin resistance (IR) and obesity were involved in the etiology of NAFLD. NAFLD is associated with obesity [4], IR [5, 6], and type 2 diabetes (T2D) [7]. Additionally, NAFLD patients with IR compared with those without IR have higher rates of elevated liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [8]. Maybe more interestingly, IR is more severe in patients with NASH in comparison to patients with simple fatty liver

[9]. Finally, NAFLD is associated with higher insulin levels [10] and insulin pathway related gene polymorphisms [11-13].

It appears that ghrelin (product of the *GHRL* gene) which exerts its actions via its receptor, the growth hormone secretagogue receptor (product of the *GHSR* gene), may have a role in NAFLD pathogenesis. Insulin secretion, IR, obesity, and NAFLD are all associated with alterations in circulating levels of ghrelin. In addition to the stomach, ghrelin is also produced in small quantities by many tissues including the hypothalamus, pituitary, small intestine, pancreas, brain, lung, heart, and kidney. Ghrelin has an important role in long-term regulation of body weight by affecting appetite and food intake, regulation of lipid and glucose metabolism, growth hormone secretion, and inflammatory processes [14–16]. Previous studies have also demonstrated that circulating levels of ghrelin were negatively associated with body mass index (BMI) [17–19], IR [17, 20, 21], T2D [20, 21], hypertension [20], and NAFLD [22]. Based on these considerations, we designed the present study to examine the possible association of *GHRL* (rs696217) and *GHSR* (rs2922126) gene polymorphisms with NAFLD.

METHODS

The present study was conducted as a case-control retrospective study and a total of 310 subjects, including 153 cases with biopsy-proven NAFLD (age range, 32–85 years) and 157 controls (age range, 30–81 years) were recruited. We did a multicenter collaborative study from April 2010 to July 2013. The diagnosis of NAFLD was made according to the following criteria: a) ultrasonographic evidence of fatty liver and high serum levels of liver enzymes (ALT, AST, GGT), b) alcohol consumption < 20 g/day in men and < 10 g/day in women, c) excluding patients with other causes of liver disease including viral hepatitis, Wilson's disease, alpha-1 antitrypsin deficiency, and use of drugs known to induce steatosis, d) histologic confirmation of NAFLD by an experienced pathologist who was unaware of the patients' clinical and biochemical data and scored biopsies using the Brunt's criteria. Steatosis and necroinflammation were graded from 0 to 3 and fibrosis was staged from 0 to 4 [23]. The controls had neither liver steatosis (examined by abdominal ultrasonography), elevated liver enzymes, nor viral hepatitis infection (examined by blood test). None of them were alcoholic or drank regularly and none were on regular medications. The control subjects were recruited from the institute staff and medical students. All 310 subjects were Iranian and genetically unrelated and informed consent was obtained from all of them before their participation in the study. Information on demographic, anthropometric, and clinical characteristics of the cases and controls was recorded using a self-administered questionnaire before diagnosis of NAFLD. Body mass index of each subject was calculated as the body weight divided by height squared (kg/m^2). This study was approved by the Ethical Committee of the Institute and it was conducted according to the Helsinki Declaration.

Genomic DNA was isolated from 5ml EDTA-anti-coagulated whole blood using standard methods, and *GHRL* rs696217 and *GHSR* rs2922126 gene polymorphisms were genotyped using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. The criteria for selecting these two polymorphisms were their common use in the previous studies and their position in the gene (exon or promoter). Laboratory personnel who performed the genotyping were blinded to the subjects' clinical data including their case or control status. Characteristics of the studied gene single nucleotide polymorphisms (SNPs) and PCR and RFLP conditions are summarized in Table I. The PCR products were digested overnight with the appropriate restriction enzymes (Fermentas, Leon-Rot, Germany) and the RFLP products were run on 3.0–3.5% agarose gels and then stained with ethidium bromide for visualization under ultraviolet light. The *GHRL* and *GHSR* genotypes of each subject were identified according to the digestion pattern and the presence or absence of the BseNI or BsrBI sites, respectively. For quality control reasons, we repeated the genotyping analysis of approximately 20% of all the subjects, and all the results were concordant.

Differences in demographic, anthropometric or clinical factors were calculated using chi-square (χ^2) test or t-test when appropriate. χ^2 test was also used to examine the differences in the allele frequencies. We used logistic regression analysis for comparing the distribution of the genotype frequencies and for adjusting confounding factors too. The odds ratios (OR) which present the measure of associations were given with the respective 95% confidence intervals (CI). All the statistical analyses were conducted using SPSS software for Windows, version 25.0 (SPSS Inc. Chicago, IL, USA). Significance was assumed for $p<0.05$.

RESULTS

Demographic, anthropometric, clinical, and biochemical characteristics of the study population are presented in Table II. The cases with NAFLD were older and had a higher BMI than the controls ($p<0.001$). The percentages of males and smokers were also higher in the cases ($p<0.001$ and $p=0.011$,

Table I. Studied variants in ghrelin (*GHRL*) and ghrelin receptor (*GHSR*) genes

Gene (SNP) Location (Base change)	Primer sequence (forward and reverse)	Annealing temperature	PCR product size (bp)	Restriction enzyme	RFLP products size (bp)
GHRL (rs696217) Exon 2 (G/T)	5'-TTTATAGTTCTGGGGAGCTTG-3' 5'-TGTTTCCCCCTTCCAGCAGAG-3'	62 °C	254	BseNI	T: 254 G: 130+124
GHSR (rs2922126) Promoter (T/A)	5'-TCCCAGTCCTTTGTATCACTCG-3' 5'-TTATGCCTCAAAAAATGTTTCCCG-3'	64 °C	218	BsrBI	T: 218 A: 194+24

GHRL: ghrelin; GHSR: ghrelin receptor; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism.

Table II. Study population characteristics[a]

Variables	Controls (n=157)	NAFLD patients (n=153)	p
Age (years)	28.9 (7.1)	38.1 (9.2)	<0.001
BMI (kg/m^2)	23.6 (3.1)	29.4 (5.3)	<0.001
Gender			
Men	84 (53.5)	109 (71.2)	
Women	73 (46.5)	44 (28.8)	<0.001
Smoking status			
Never smoker	144 (91.7)	114 (74.5)	
Former smoker	9 (5.7)	20 (13.1)	
Current smoker	4 (2.6)	19 (12.4)	0.011
SBP (mmHg)	114.7 (13.4)	124.1 (15.7)	<0.001
DBP (mmHg)	69.3 (8.6)	74.4 (9.5)	<0.001
AST (IU/L)	19.3 (7.4)	39.2 (17.4)	<0.001
ALT (IU/L)	19.2 (10.7)	72.0 (41.1)	<0.001
GGT (IU/L)	18.3 (8.8)	57.4 (32.6)	<0.001
Steatosis			
Grade 1		40 (26.1)	
Grade 2		82 (53.6)	
Grade 3		31(20.3)	
Necroinflammation			
Grade 0		48 (31.4)	
Grade 1		61 (39.9)	
Grade 2		42 (27.5)	
Grade 3		2 (1.3)	
Fibrosis			
Stage 0		88 (57.5)	
Stage 1		58 (37.9)	
Stage 2		7 (4.6)	
Stage 3		-	
Stage 4		-	

[a]Variables presented as mean (SD) or number (%); ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; DBP: diastolic blood pressure; GGT: gamma glutamyl transferase; NAFLD: nonalcoholic fatty liver disease; SBP: systolic blood pressure.

respectively). Additionally, the cases had higher systolic blood pressure (SBP), diastolic blood pressure (DBP), and higher serum levels of AST, ALT and gamma glutamyl transferase (GGT) than the controls (p<0.001).

Table III summarizes the genotype and allele distributions for *GHRL* rs696217 and *GHSR* rs2922126 gene polymorphisms in the cases with NAFLD and the controls. The *GHRL* rs696217 "GT+TT" genotype compared with the "GG" genotype, and the "GT" genotype compared with the "GG" genotype occurred less frequently in the cases than the controls (p=0.018; OR=0.35, 95%CI: 0.14–0.84 and p=0.046; OR=0.4, 95%CI: 0.16–0.98, respectively). These differences remained significant even after adjustment for confounding factors including age, BMI, gender, smoking status, SBP, and DBP. In addition, the *GHRL* rs696217 'T' allele compared with 'G' allele was significantly underrepresented in the cases with NAFLD compared to controls (p=0.007; OR=0.33, 95%CI: 0.15-0.76). However, we observed no significant difference in genotype or allele frequencies between the cases with NAFLD and the controls for *GHSR* rs2922126 gene polymorphism either before or after adjustment for confounding factors.

DISCUSSION

We designed this study to explore the possible association between *GHRL* and *GHSR* gene polymorphisms and NAFLD. The *GHRL* rs696217 "GT+TT" genotype compared with the "GG" genotype, and the "GT" genotype compared with the "GG" genotype, as well as the *GHRL* rs696217 'T' allele compared with 'G' allele appeared to be a marker of decreased NAFLD susceptibility. However, we observed no significant association between the *GHSR* rs2922126 gene polymorphism and NAFLD.

Nonalcoholic fatty liver disease such as other complex multifactorial disorders results from the interaction between

Table III. The genotype and allele frequencies of ghrelin (GHRL) and ghrelin receptor (GHSR) gene polymorphisms in the cases with nonalcoholic fatty liver disease (NAFLD) and in controls[a]

Gene (Variant)	Controls (n=157)	NAFLD patients (n=153)	OR (95% CI)	p[b]
***GHRL* (rs696217)**				
Genotype-wise comparison				
GG	116 (73.9)	136 (88.9)	1.0 (reference)	
GT	35 (22.3)	17 (11.1)	0.40 (0.16–0.98)	0.046
GT and TT	41 (26.1)	17 (11.1)	0.35 (0.14–0.84)	0.018
Allele-wise comparison				
G	267 (85.0)	289 (94.4)	1.0 (reference)	
T	47 (15.0)	17 (5.6)	0.33 (0.15–0.76)	0.007
***GHSR* (rs2922126)**				
Genotype-wise comparison				
TT	83 (52.9)	88 (57.5)	1.0 (reference)	
TA	33 (21.0)	24 (15.7)	1.74 (0.55–5.51)	0.346
AA	41 (26.1)	41 (26.8)	0.45 (0.12–1.69)	0.234
TA and AA	74 (47.1)	65 (42.5)	0.99 (0.39–2.54)	0.986
AA versus others	41 (26.1)	41 (26.8)	0.39 (0.11–1.42)	0.151
Allele-wise comparison				
T	199 (63.4)	200 (65.4)	1.0 (reference)	
A	115 (36.6)	106 (34.6)	0.80 (0.49–1.29)	0.359

[a] Variables presented as number (%); [b] Adjusted for age, body mass index, gender, smoking status, systolic blood pressure and diastolic blood pressure in genotype-wise comparisons.

many environmental and genetic factors [24]. Therefore, it could be difficult to discover the majority of genes involved in its pathogenesis due to the fairly small individual effects and complex interactions of these genes. Nevertheless, studying polymorphisms in candidate genes is one of the reliable approaches to identifying novel susceptible genes in such diseases. Previous studies have shown that NAFLD shares genetic associations with metabolic syndrome-associated disorders, including T2D, obesity, cardiovascular diseases and dyslipidemia. This suggests that they have common etiology and share similar pathogenic mechanisms [25-27]. The genes involved in IR, obesity, and inflammation are potential candidate genes for NAFLD. On the other hand, there is accumulating evidence that ghrelin may be involved in the pathogenesis of NAFLD owing to its different physiological functions and its role in IR, obesity, and inflammation. NAFLD patients have a lower circulating level of ghrelin [22] and a possible hypothesis for that is obesity-related hyperinsulinemia and the inhibitory effect of insulin on ghrelin secretion from the X/A-like cells of the stomach [28]. In accordance with this hypothesis, a previous study has reported that insulin might be an independent modulator of ghrelin levels, and insulin mediated the effects of nutritional status and chronic energy balance on ghrelin levels [29].

The first gene studied here, *GHRL*, is a polymorphic gene with more than 200 SNPs and is located on the short arm of chromosome 3 and encodes ghrelin protein, a 28-amino acid gastric peptide. Considering the wide variety of biological functions, any defects in *GHRL* gene may lead to obesity, IR, and inflammation that are involved in the development and progression of NAFLD. As mentioned earlier, we found that the *GHRL* rs696217 "GT+TT" genotype and "GT" genotype compared with "GG" genotype, as well as "T" allele compared with "G" allele had a protective effect for NAFLD susceptibility. The rs696217 variant is characterized by a G to T substitution in exon 2 of the *GHRL* gene resulting in a leucine-to-methionine exchange at position 72 of the C-terminal tail of the preproghrelin protein. This SNP does not change the sequence of the mature ghrelin, but it may result in the alterations in the mRNA stability or protein processing, which in turn might modify ghrelin secretion and/or activity [30]. The Leu72Met polymorphism influences the expression of the *GHRL* gene [31]. The molecular mechanism through which the rs696217 variant may affect the function of *GHRL* gene and lead to NAFLD is not clear. However, a possible hypothesis is that the rs696217 "G" allele of the *GHRL* gene causes a defect in the *GHRL* gene somehow, which in turn decreases ghrelin levels in the patients with NAFLD, and in this way plays a role in the pathogenesis of NAFLD through obesity, IR, or/and inflammation. Such a mechanism is speculative at the present but biologically plausible and previous studies are in line with this hypothesis. The circulating level of ghrelin is inversely associated with BMI [17–19], and it is increased after weight loss [32]. Ghrelin also stimulates growth hormone release, which in turn leads to inhibited synthesis of fatty acids and decreased adiposity through the known lipolytic

activity of growth hormone [33]. Furthermore, ghrelin which is expressed in pancreatic islet beta-cells [34], plays a major role in the modulation of insulin signaling pathway and inhibition of glucose-stimulated insulin secretion [35, 36]. Previous studies have also reported significant negative correlations between ghrelin levels and insulin levels [18, 20], hyperinsulinemia [18], IR [17, 20, 21], and T2D [20, 21]. Insulin resistance expedites the release of free fatty acids from adipose tissue and its influx into liver [5, 6, 37]. Insulin resistance is an independent predictor of advanced liver fibrosis too [38]. The other mechanism linking ghrelin with the pathogenesis of NAFLD is through inflammation. Ghrelin has anti-inflammatory and antioxidant activities [39, 40] and it suppresses the expression of inflammatory cytokines [41]. The administration of ghrelin attenuates NAFLD-induced liver injury, oxidative stress, and inflammation [42]. There is also more evidence that supports the hypothesis that the "T" or Met72 allele of the *GHRL* rs696217 (Leu72Met) polymorphism has a protective effect on NAFLD. Met72Met subjects have lower values of BMI [43, 44, 45], fat mass [43], visceral fat [43], triglycerides [31], total cholesterol [46], HDL cholesterol [46], LDL cholesterol [46], insulin [31], IR [31], T2D [47], and a higher ghrelin level [31, 48]. These findings are in agreement with ours. Additionally, it has been suggested that other *GHRL* gene polymorphisms are significantly associated with circulating levels of glucose [49], insulin [46], metabolic syndrome [50], hypertension [51], and hepatocellular carcinoma [52]. And finally, serum levels of ghrelin decrease in the patients with either liver advanced fibrosis or liver cirrhosis and *GHRL* gene variants influence the progression of liver fibrosis and liver cirrhosis [53, 54]. The other possible hypothesis linking the rs696217 polymorphism with NAFLD risk is through linkage disequilibrium. Rs696217 may not be a functional polymorphism; instead, it might be in complete or near-complete linkage disequilibrium with a yet unknown functional variant of *GHRL* gene.

The other gene studied here, *GHSR*, is located on the long arm of chromosome 3 and encodes *GHSR* protein. In this study, we found no significant association between the *GHSR* gene rs2922126 polymorphism and NAFLD susceptibility. This polymorphism is located in the promoter region and alterations in promoter sequence may affect the expression and function of the *GHSR* protein. Previous studies have reported that *GHSR* rs2922126 and rs572169 variants are associated with circulating glucose levels [55], obesity [56] and metabolic syndrome [55]. Notwithstanding the biological plausibility, we suggest that *GHSR* is not a predisposing gene for NAFLD. As stated, in complex diseases, such as NAFLD, the effect of a majority of genes may not be easy to identify due to their modest individual effects and complex interactions [57]. Thus, to conclude, the *GHSR* gene does not play a role in the pathogenesis of NAFLD, the rs2922126 and other *GHSR* gene polymorphisms should be investigated in other populations.

Some limitations of this study should be addressed when interpreting the findings. One limitation was the modest sample size that precluded us from performing detailed analyses. If we had a larger study population, we would have been able to perform multiple sub-analyses too, for example the evaluation of the association between the *GHRL* and *GHSR* gene polymorphisms and the risk of simple fatty liver and NASH separately. Another limitation was the lack of information on serum levels of ghrelin which was due to budget limitations. The other limitation was that by testing only one variant in each gene, the coverage of the genes was incomplete. And finally, the cases and controls were not matched by age and sex. Notwithstanding these limitations, this case-control study was well designed, and liver biopsy was used as the gold standard method for confirming the diagnosis of NAFLD. It provided interesting novel information that was in concordance with previous publications too.

CONCLUSIONS

This study showed, for the first time, that the *GHRL* Leu72Met polymorphism might be a genetic contributor to NAFLD susceptibility. This observation is relevant from a theoretical standpoint; however, our findings warrant further investigations in other populations.

Conflicts of interest: None to declare.

Authors' contributions: S.P.T., M.S., G.R., R.D., H.N., A.A., H.F., F.M.G and M.R.Z. performed the analyses, collected the data and drafted the manuscript. S.P.T coordinatated the study. T.M. conceived and designed the study, drafted the manuscript, analysed the data and supervised the project.

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