



Leu72Met polymorphism of *GHRL* gene increase the risk factor of obesity in a Javanese ethnic group from Indonesia

Demes Chornelia Martantiningtyas^{a,b,*}, Pramudji Hastuti^b, Ahmad Hamim Sadewa^b

^a Department of Biochemistry, Faculty of Medicine, Maranatha Christian University, Indonesia

^b Department of Biochemistry, Faculty of Medicine, Universitas Gadjah Mada, Indonesia

ARTICLE INFO

Keywords:

Ghrelin
Obesity
Insulin secretion
Leu72Met

ABSTRACT

Purpose: Ghrelin is found as an endogenous ligand for growth hormone secretagogue receptor (GHSR). Several studies have reported an association between plasma ghrelin and GHRL-single nucleotide polymorphism, namely the Leu72Met polymorphism, with Body mass Index, type 2 diabetes mellitus, and insulin resistance. The role of gene variant of GHRL Leu72Met in obesity is still unclear. The aim of the present study was to examine the associations of Leu72Met with ghrelin levels, insulin secretion, and obesity among Javanese subjects.

Materials and methods: All subjects were measured with anthropometry, and fasting blood glucose was measured by the glucose oxidase-phenol and 4 aminophenazone method. Plasma insulin and ghrelin were measured using ELISA. Insulin secretion was calculated using HOMA analysis. The Leu72Met genetic variant of the ghrelin gene was screened using PCR-RFLP.

Results: Plasma ghrelin concentrations were lower in the obese group than the lean group ($P < 0.05$). Fasting plasma ghrelin was negatively correlated with body mass index and insulin secretion. Subjects with the Met72 allele carried a higher risk of obesity than the subjects with the Leu72 allele (OR = 4.928 [95% CI = 2.424–10.01]).

Conclusion: The conclusion of this study is Leu72Met polymorphisms increases the risk of obesity in Javanese ethnicity, but this polymorphism does not play a role in plasma ghrelin secretion and plasma insulin secretion.

1. Introduction

Obesity is a problem for millions of people worldwide. Obesity is known as a multifactorial disease, controlled by genetic and environmental factors (Hetherington and Cecil, 2010). Each individual will respond to different obesogenic environments, most likely related to gene polymorphism (Rudkowska and Pérusse, 2012). Identification of genetic variant associated with obesity will help predict a person's genetic risk for obesity and can be used as "personalized" genetic for the prevention or treatment of obesity. There are approximately 118 genes involved in obesity (Jiao et al., 2008). Ghrelin is a orexigenic hormone that plays a role in regulating food intake, adiposity, the secretion of growth hormones and energy balance (Kanoski et al., 2013). Plasma ghrelin levels in obese subjects are lower compared to groups with normal weight. Low plasma levels of ghrelin are associated with hypertension, insulin resistance and Type 2 diabetes (Shiyya et al., 2009).

The gene encoding ghrelin is GHRL, located on the short arm of Chromosome 3 (3p25-26) (Seim et al., 2007). Polymorphism in the

ghrelin gene can cause protein disruption or inactivation, changes in growth hormone secretion and disturbances in energy balance (Liu et al., 2012). Leu72Met polymorphism has been associated with obesity, insulin secretion, and eating disorders (Tschöp et al., 2000).

The role of gene variant of GHRL Leu72Met in obesity is still unclear. Gene Variant of Leu72Met was associated with obesity in children in Italy (Miraglia del Giudice et al., 2004), overweight males in Japan (Kuzuya et al., 2006), insulin resistance in obese individuals, and central obesity and metabolic syndrome in men in a Spanish population (Mora et al., 2015). In contrast, other research concerning GHRL Leu72Met gene polymorphism did not associate it with obesity in a population of Denmark (Larsen et al., 2005), nor with Type 2 diabetes in a population of Korea [19]. Research on gene variant of GHRL Leu72Met showed different results and has been inconsistent in various ethnic groups causing this research of genetic polymorphism to become more interesting to study. The aim of the present study was to examine the associations of Leu72Met with ghrelin levels, insulin secretion, and obesity among Javanese subjects.

* Corresponding author at: Department of Biochemistry, Faculty of Medicine, Maranatha Christian University, Jl. Surya Sumantri No 65, Indonesia.

E-mail address: dchornelia@yahoo.com (D.C. Martantiningtyas).

<https://doi.org/10.1016/j.mgene.2021.100912>

Received 13 September 2020; Received in revised form 28 March 2021; Accepted 30 April 2021

Available online 5 May 2021

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2. Methods and materials

2.1. Subjects

A case-control study in which were included one hundred obese subjects and ninety-eight normal weight control subjects. Obese subjects were determined based on BMI cut-off value for obesity in Indonesia. Body mass index (BMI) of values equal to or greater than 25 kg/m² is categorized as obese and non-obese subjects with BMI 18.5–22.9 kg/m². Exclusion criteria in this study: Non-ethnic Javanese, Aged more than 35 years, have a disease associated with the metabolic syndrome, weight loss program, smoking, fasting blood sugar ≥ 125 mg/dL.

2.2. Anthropometric measurements

Weights were measured to the nearest 0.1 kg using calibrated balances or electronic scales. Heights were measured to the nearest 1 mm. Body mass index (BMI) was calculated as weight divided by height squared (kilograms per square meter).

2.3. Biochemical analyses

Fasting blood glucose estimated by using GOD-PAP (Glucose Oxidase – Peroxidase Aminoantipyrin) enzymatic colorimetric test. Plasma insulin levels was measured by Enzyme-Linked Immunosorbent Assay (ELISA) method, applied according to manufacturer's protocol (DRG® Iso-Insulin ELISA Kit). Plasma ghrelin levels was measured by ELISA method, applied according to manufacturer's protocol (Human GHRL (Ghrelin) ELISA kit-Elabscience). Expression function of β -cells of the pancreas in insulin secretion was determined using the homeostasis model assessment of β -cell function (HOMA- β), with the formula: $\text{HOMA-}\beta = (20 \times \text{fasting insulin } (\mu\text{IU} / \text{mL}) / (\text{fasting glucose } (\text{mmol} / \text{L}) - 3.5)$.

2.4. DNA extraction and genotyping

Genomic DNA from buffy coat was extracted by Wizard® Genomic DNA Purification Kit, following the manufacturer procedures. The polymerase chain reactions were carried out using the PCR System 9600 thermal cycler Esco according to standardized laboratory protocols. Amplification uses forward primer 5'GCTGGGCTCCTACCTGAGC-3' and primary reverse 5'-GGACCCTGTTCACTGCCAC-3' (Gohar et al., 2012). The total reaction volume used was 25 μ L, including 2 μ L of DNA, 1 μ L of forward primer, 1 μ L of reverse primer, 12.5 DreamTaq Green PCR Master Mix, and 8.5 μ L free deionized water nuclease. The PCR process starts with an initial denaturation of 94 °C for 5 min, followed by 35 denaturation cycles of 94 °C for 1 min, Annealing 65 °C for 1 min, elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The resulting PCR product is 618 bp. The amplified fragments by PCR were incubated for enzyme digestion with 1.5 U of *Bsr*I restriction enzyme. The digested PCR fragments along with DNA ladder were resolved by electrophoresis using 3% agarose gel. RFLP products produced for Leu72Leu = 517 + 101 bp, Leu72Met = 618 + 517 + 101 and Met72Met = 618 bp.

2.5. Statistical analyses

Data were analyzed using SPSS computer software program with a significance level of 5% ($p < 0.05$). Normality of data using the Kolmogorov-Smirnov test. Mean plasma insulin and plasma ghrelin in obese group and controls were compared using unpaired *t*-test when the data are normally distributed. To compare the mean plasma insulin and plasma ghrelin between genotype groups performed one-way ANOVA test, then the test continued with Post-Hoc. Differences in the genotype and allele frequencies in each case and control groups were analyzed using Chi-square analysis, alternatively, use the Fisher-Exact test.

3. Results

3.1. Basal characteristics of the study groups

Basal characteristics of the study groups are shown in Table 1. The mean HOMA- β in the control and obese groups was not significantly different ($P = 0.219$). Fasting plasma ghrelin levels in this study were measured in obese subjects and control subjects. Comparison of the average between obese and control subjects (Table 1) showed that plasma ghrelin levels were lower in obese subjects (1.06 ± 0.23 ng / mL) compared with control subjects (1.36 ± 0.50 ng / mL), and was statistically significant ($P < 0.001$).

3.2. Association Leu72Met Polymorphism with BMI, plasma ghrelin, plasma glucose, plasma insulin and HOMA- β

The data in Table 2. show a significant difference (indicate *p*-value) for the body mass index of the genotype group. The statistical results of plasma Ghrelin, plasma glucose, plasma insulin and HOMA- β , were not significant between the genotype groups, but there were mean differences. Plasma Ghrelin in the CC (Leu72Leu) genotype was higher (1.37 ± 0.53 ng / mL) than CA (1.29 ± 0.244 ng / mL) in the control group. HOMA- β in the CC genotype (92.99 ± 35.5 ng / mL) was lower than the CA genotype (99.80 ± 37.7 ng / mL) in the control group. Obese groups of plasma ghrelin levels in the CC genotype (Leu72Leu) are higher than mutant genotypes, as well as plasma insulin levels, plasma glucose, and HOMA- β .

3.3. Genotype distribution

The genotype distribution was statistically analyzed using chi-square. The wild type genotype was found to be 87.8% in control and 50% in the obese group. The mutant genotype AA (Met72Met) was only found in of 7.8% of obese group. CA mutant genotypes (Leu72Met) were found in 12.2% of the control group and in 42.2% of the obese group.

Comparison of genotype frequencies between controls and obese groups were highly significant different ($p < 0.001$). In the control and obese groups A were 10.1% and 35.6% with an odds ratio of 4.928. The odds ratio of genotype AA + CA to CC is calculated using Yate correction. The OR of genotype AA + CA to CC was 4.96 (95% CI = 2430–10,125) and highly significant different ($P < 0.001$) (Table 3).

4. Discussion

The mean comparison between obese and control subjects ghrelin levels (Table 1.) showed that plasma ghrelin levels were lower in the obese subjects (1.06 ± 0.23 ng / mL) compared with control subjects (1.36 ± 0.50 ng / mL), and was statistically significant ($P < 0.001$). The Pearson correlation, ghrelin and body mass index were negatively

Table 1
Demographic, clinical and anthropometric characteristics of the study groups.

	CS n = 98	OS n = 102	p
Demographic			
Age (years)	21.14 $\pm$ 3.861	22.06 $\pm$ 4.085	0.105
Female n (%)	51 (52)	49 (48)	NS
Male n (%)	47 (48)	53 (52)	NS
Clinical and anthropometric			
BMI (kg/m ²)	20.55 $\pm$ 2.185	31.05 $\pm$ 4.109	< 0.001*
Fasting glucose (mg/dL)	88.67 $\pm$ 11.47	93.35 $\pm$ 14.88	0.014*
Ghrelin Plasma (ng/mL)	1.36 $\pm$ 0.50	1.06 $\pm$ 0.23	< 0.001*
HOMA- β	93.83 $\pm$ 35.72	101.32 $\pm$ 48.92	0.219

CS Control Subjects, OS Obese Subjects. Values are reported as percentages or means $\pm$ SD. Normality test data using Kolmogorov-Smirnov test: $P < 0.05$, normally distributed data. *Test Independent *t*-test: $P < 0.05$, significant difference.

Table 2

Levels of ghrelin plasma, insulin, glucose and HOMA- β in control and obese subjects with genotype Leu72Leu (CC), Leu72Met (CA), and Met72Met (AA).

Genotype	CS n = 98			OS n = 102		
	CC n = 86	CA n = 12	p	CC n = 51	AA+CA n = 51	p
BMI	20.38 ± 2.21	21.76 ± 1.51	0.039*	30.27 ± 3.95	31.87 ± 4.14	0.049*
Ghrelin plasma (ng/mL)	1.37 ± 0.53	1.29 ± 0.244	0.630	1.08 ± 0.248	1.05 ± 0.232	0.689
Fasting glucose (mg/dL)	88.58 ± 11.5	88.50 ± 11.0	0.833	92.39 ± 14.13	94.36 ± 15.8	0.506
Fasting Insulin (μIU/mL)	5.74 ± 1.27	6.59 ± 2.37	0.058	6.87 ± 1.76	7.07 ± 1.84	0.604
HOMA-B	92.99 ± 35.5	99.80 ± 37.7	0.539	100.54 ± 43.6	102.65 ± 54	0.862

CS Control Subjects, OS Obese Subjects.

* Statistically significant P values <0.05 are indicated.

correlated ($r = -0.314$) and were statistically significant ($P < 0.001$). These data are consistent with previous studies (Shiyya et al., 2002; Tschoep et al., 2001) reporting fasting plasma ghrelin found to be low in obese subjects compared with subjects with normal weight. The mechanism of the ghrelin response found low in obese individuals is not entirely clear. Low plasma ghrelin levels may be a consequence of excess energy, this opinion is evidenced by the increase in plasma ghrelin levels in Obese subjects who experience weight loss. Changes in plasma ghrelin levels in the blood circulation level may be reflects the imbalance of regulatory factors or mechanisms responsible for ghrelin secretion.

Abnormalities of plasma ghrelin levels in obese subjects are still not clear as to cause or effect, however ghrelin gene polymorphisms and their receptors involved in the incidence of obesity (Ukkola, 2011). Obesity can cause hyperactivity of the endocannabinoid system that plays a role in the regulation of food intake. The endocannabinoid system hyperactivity may inhibit ghrelin secretion. Endogenous endocannabinoids inhibit cannabinoid receptor type 1 (CB1) present in alimentary duct. Inhibition of cannabinoid type 1 receptor (CB1) will inhibit ghrelin secretion from gastric fundus cells (Cuellar and Isokawa, 2011).

Obese individuals do not experience ghrelin disruption or response, or disruption in ghrelin transport through the blood brain barrier (BBB), because peripheral ghrelin administration can still increase hunger in obese subjects (Al Massadi et al., 2011). Obese patients may be more sensitive to ghrelin, for example over expression of growth hormone secretagogue receptor (GHS-R). The effect of ghrelin on insulin secretion in this study was also seen. Insulin secretion was found to be based on HOMA- β , the mean comparison was lower in the control group (93.83 ± 35.72) than in the obese group (101.32 ± 48.92), but not statistically significant ($P = 0.219$) mean elevation of HOMA- β , followed by low plasma ghrelin.

Table 3

Distribution of genotypes (CC, CA, AA) and allele (A, C) in the gene polymorphism GHRL Leu72Met.

Leu72Met	CS n = 98	OS n = 102	OR (CI 95%)	p	H-W equation	P
AA	–	8 (7.8%)			135	0.959
CA	12 (12.2%)	43 (42.2%)	2.043 (1.768–2.360)	<0.001	59	
CC	86 (87.8%)	51 (50.0%)			6	
AA+CA	12 (12.2%)	51 (50%)	4.960 (2.430–10.125)	<0.001*		
CC	86 (87.8%)	51 (50%)				
A	11 (10.1%)	52 (35.6%)	4.928 (2.424–10.01)	<0.001*		
C	98 (89.9%)	94 (64.4%)				

CS Control Subjects, OS Obese Subjects.

* Chi-square test: $p < 0.05$; significant difference.

The ghrelin gene is one of the gene candidates associated with obesity. Leu72Met variation is a variant of the pre-proghrelin gene, which undergoes changes in the amino acid leucine at number 72 to methionine. The frequency of these variant alleles ranges from 1 to 9% in different populations (Ukkola, 2011). Carriers of the Met72 allele are associated with obesity (Korbonits et al., 2004), early obesity (Miraglia del Giudice et al., 2004), and a history of obesity in a family (Vivenza et al., 2004). Leu72Met variation is associated with high body mass index in Japanese men but not in women or older subjects (Kuzuya et al., 2006). Case study control (obese and non obese) European cohorts, also showed Leu72Met variant more easily become obese (Gueorguiev and Korbonits, 2013). Studies in Korean populations with a study group of 500–1500 subjects identified an association between Leu72Met and obesity and were statistically significant (Kim et al., 2016). Supporting previous research the value of odds ratio of CA (Leu72Met) + AA (Met72Met) to CC (Leu72Leu) to 4.96 (95% CI = 2.4–10.12) was calculated using Yates correction. This value shows that CA + AA genotype carriers have a 4.9 times risk of becoming obese compared to CC genotype carriers. Individuals carrying the A allele (Met72) are more receptive than carriers of C allele (Leu72) in environments that promote weight gain. Allele A carriers (Met72) are associated with early obesity at a young age while in an environment that triggers weight gain (Liu et al., 2011). In this study, individuals carrying two alleles A (OR = 2.043; 95% CI = 1.768–2.360) had a higher risk of becoming obese than carrying only one allele A (OR = 0.17; 95% CI = 0.80–1, 34). The data about Met72 allele carriers are more susceptible to obesity, can be used as one of the targets for obesity therapy.

Ghrelin and HOMA- β levels were obtained in this study compared between each genotype to see the effect Leu72Met polymorphism with changes in ghrelin concentration and insulin secretion in the control and obese groups. Genotype of carrier A allele (Met72) in the group controls and obesity showed a decrease in ghrelin plasma levels, although statistically insignificant. Gene Variant on the codon 72 preproghrelin gene (Leu72Met) is located outside the area to encode ghrelin mature production. Gene Variant that occurs does not cause it changes in ghrelin mature sequence, but produce changes in the stability of messenger RNA or the processing protein that causes modification of ghrelin secretion or its activity without changing circulation concentration ghrelin (Kim et al., 2016). Changes in plasma ghrelin secretion will affect energy balance, this is evidenced by the increase in body mass index in individuals carrying Met72 alleles (in the obesity group = 31.87 ± 4.14 kg / m²; in the control group = 21.76 ± 1.51 kg / m²). Insulin secretion between groups genotype shows differences not statistically significant, even though the Met72 carrier allele showed an increase in HOMA- β compared to wild type (Table 3). Plasma insulin levels and fasting glucose were also found to be insignificant between genotype groups, this result is the same as the research conducted by Takezawa et al. and Gohar et al. (Gohar et al., 2012; Takezawa et al., 2009). Different results shown by Korbonits et al., Met72 allele carriers experience decreased insulin secretion after OGTT administration (Korbonits et al., 2004). Contradiction in the results of influence genotype in insulin secretion, may be influenced by race and living environment each individual, so

that it can cause different results.

The genotyping of GHRL Leu72Met and PCR-RFLP genotyping genes was found in three genotypes: CC (Leu72Leu) of 50%, CA (Leu72Met) of 42.2%, and AA (Met72Met) of 7.8% in obese group. Two CC (Leu72Leu) genotypes were 87.8% and CA (Leu72Met) were 12.2%, found in the control group. This result is similar to that of Imaizumi et al., the ratio of Leu72Met in control and obese significantly (Imaizumi et al., 2016). Investigations performed by Kim's in obese children in Korea showed Leu72Met genotype frequency of 35.40% and showed no significant difference between obese and controls. In the study of Hinney et al. the frequency of Leu72Met was 9.10% and showed a non-significant difference between obese subjects and controls (Hinney et al., 2002). Following this discovery, the Leu72Met frequency of the ghrelin gene is different, related to race and area.

The distribution of the genotypes in this study were analyzed using the Chi-square test to compare with the expected results according to the balance of Hardy Weinberg (H-W). The Hardy-Weinberg principle states that the allele and genotype frequencies in a population will not change from one generation to another in H-W equilibrium. The results of the Hardy-Weinberg (H-W) equilibrium statistical test on the Leu72Met polymorphism genotype of the GHRL gene in the Javanese population showed insignificant differences between the distribution of the genotype results of the study and the H-W population ($P = 0.959$). This means that the CC, CA, and AA genotypes in this study are in accordance with the distribution of the genotypes in the population or are in equilibrium.

5. Conclusion

The conclusion of this study is Leu72Met polymorphisms increases the risk of obesity in Javanese ethnicity, but this polymorphism does not play a role in plasma ghrelin secretion and plasma insulin secretion.

Funding

This research was supported by grants Dirjen Dikti of Indonesia with the contract number 632 / UN1 1-P III / DIT-LIT / 2016 for the subject sample and measuring ghrelin levels.

Availability of data and materials

All data and materials are available with the corresponding author in the Departement of Biochemistry, Faculty of Medicine, Maranatha Christian University.

Ethical clearance

This study has received approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University with a reference number KE / FK / 532 / EC / 2016. Two hundred participants involved in the study, and all participants have signed informed consent.

Consent for publication

Have consent to publish.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

This research was supported by grants Dirjen Dikti of Indonesia with the contract number 632 / UN1 1-P III / DIT-LIT / 2016 for the subject sample and measuring ghrelin levels. We also want to thank Dr. Pramudji Hastuti Apt., M. Kes. Which is helpful in giving funds to the

detection of gene Variant of the GHRL Leu72Met. Appreciation is also given to dr.Ahmad Hamim Ph.D. on the advice and assistance in the preparation of this manuscript.

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