J Med Sci
Volume 44, No.2, June 2012: 231-238

The association of single nucleotide polymorphism (SNP) rs2922126 within ghrelin and growth hormone secretagogue receptor 1a (GHSR1a) gene with insulin resistance in obese female adolescents in Yogyakarta Special Region

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ABSTRACT

Previous study reported that among 79 obese female adolescents in Yogyakarta Special Region, 44 (55.7%) of them have insulin resistance. However, no significant differences on dietary habits and physical activity between the obese female who have insulin resistance and those who are insulin sensitive were observed. Therefore, it was thought that genetic factors are involved in the occurrence of insulin resistance. Ghrelin *and growth hormone secretagogue receptor* (GHSR) genes have been associated with the insulin signaling pathway with implications in insulin resistance. The study aimed to analyze the association between SNP (single nucleotid polymorphism) rs2922126 in GHSR1a gene with insulin resistance in obese female adolescents in Yogyakarta Special Region. Seventy eight obese female adolescents who were selected in the previous study were involved in this study. Secondary data including name of subjects, age, body height, body weight, BMI (body mass index), fasting glucose level, fasting insulin level, waist circumference and HOMA-IR index were obtained from previous study. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) methods were used to the genotype analysis of SNP rs2922126. Chi-square test was used to calculate odds ratio on genotype and allele of SNP rs2922126 GHSR1a gene in insulin resistance and insulin sensitive groups. The results showed that A/A genotype individuals in SNP rs2922126 had higher risk to develop insulin resistance, compared to A/T and T/T genotypes individuals (OR: 2.03; 95%CI: 0.54-7.57). However, it was not significantly different ($p>0.05$). Individuals with A/A genotype and A allele carriers at SNP rs2922126 tended to have a higher value of BMI, fasting glucose level, fasting insulin level, HOMA-IR, and waist circumference compared to other carriers, although it was not significant ($p>0.05$). It can be concluded that SNP rs2922126 in GHSR1a gene is not associated with insulin resistance in obese female adolescents in Yogyakarta Special Region.

ABSTRAK

Dari penelitian sebelumnya dilaporkan diantara 79 remaja putri dengan kelebihan berat badan di Daerah Istimewa Yogyakarta, 44 (55.7%) diantara mereka mengalami resistensi insulin. Namun demikian, tidak ada perbedaan dalam hal pola makan dan aktivitas fisik antara remaja putri

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dengan kelebihan berat badan yang mengalami resistensi insulin dan sensitif insulin. Oleh karena itu diduga faktor genetik berperan timbulnya resistensi insulin. Gene *ghrelin and growth hormone secretagogue receptor* (GHSR) telah dikaitkan dengan jalur sinyal insulin dengan implikasi terjadinya resistensi insulin. Penelitian ini bertujuan mengkaji hubungan antara SNP (*single nucleotid polymorphism*) rs2922126 gene GHSR1a dengan resistensi insulin pada remaja putri dengan kelebihan berat badan di Daerah Istimewa Yogyakarta. Tujuh puluh delapan remaja putri dengan kelebihan berat badan hasil seleksi dari penelitian sebelumnya terlibat dalam penelitian ini. Data sekunder yakni nama subjek, umur, tinggi badan, berat badan, IMT (indeks masa tubuh), kadar gula darah puasa, kadar insulin puasa, lingkar pinggang dan indeks HOMA-IR diperoleh dari penelitian sebelumnya. Metode *Polymerase Chain Reaction* (PCR) dan *Restriction Fragment Length Polymorphism* (RFLP) digunakan untuk analisis genotipe SNP rs2922126. Uji *Chi-square* digunakan untuk menghitung *odds ratio* pada genotipe dan alel SNP rs2922126 gene GHSR1a pada kelompok resisten dan sensitif insulin. Hasil penelitian menunjukkan individu dengan genotipe A/A pada SNP rs2922126 mempunyai risiko mengalami resistensi insulin lebih tinggi dibandingkan dengan individu dengan genotipe A/T dan T/T (OR: 2,03; 95%CI: 0,54-7,57). Namun demikian risiko ini tidak berbeda nyata ($p>0.05$). Individu dengan genotipe A/A dan alel A pada SNP rs2922126 cenderung memiliki nilai IMT, kadar gula darah, kadar insulin darah, lingkar pinggang dan HOMA-IR lebih tinggi dibandingkan dengan individu genotipe dan alel lain. Namun hal in juga tidak berbeda nyata ($p>0.05$). Dapat disimpulkan, bahwa SNP rs2922126 gene GHSR1a tidak berhubungan dengan resistensi insulin pada remaja putri dengan kelebihan berat badan di Daerah Istimewa Yogyakarta.

Keywords: GHSR1a gene - SNP rs2922126 - insulin resistance - female - obese

INTRODUCTION

Childhood obesity is associated with an increased risk of several metabolic complications, such as glucose intolerance, type 2 diabetes mellitus, and insulin resistance.[1] The increase of body weight causes an expansion of the adipose tissue mass and down regulation of insulin signaling in adipose tissue, skeletal muscle, and in the liver. These conditions are associated with the increase of free fatty acid release and can inhibit the insulin ability to stimulate glucose uptake by muscles and adipocytes and hepatic glucose production.[2-4]

Some factors influencing the insulin resistance development include environmental factors, physical activity and genetic factors. Many genes have been associated with the insulin signaling pathway with implications in insulin resistance such as genes encoding insulin receptor (INSR genes), insulin receptor substrat (IRS-1 and -2 genes), and ghrelin receptor gene (GHSR1a).[5,6]

Previous study showed that among 2,120 female adolescents in Junior High School in Yogyakarta Special Region who were screened for obesity, 137 of them were obese. Further study was conducted to evaluate the factors influencing the insulin resistance development. Among 79 of the obese female involved in the study, 44 of them were known to possess insulin resistance state. No significant difference in dietary habits and physical activity between obese female with insulin resistance state and non insulin resistance state was observed in the study. It was thought that genetic factors influenced the insulin resistance state of subjects in the study.[7]

Ghrelin receptor genes (GHSRs) plays an important role in regulating the process of obesity and type 2 diabetes that are important components of metabolic syndrome.[8] Human GHSR gene is located in chromosome 3 (3q26.27).[9] Two distinct transcripts of GHSR are known: one encoding the full-length G-protein-coupled receptor (GHSR1a) and the

other encoding a truncated receptor (GHSR1b). Only GHSR1a can be activated by ghrelin, while the function of GHSR1b remains unknown.[10] GHSR1a on mRNA level is expressed in many tissues, such as pituitary, thyroid, pancreas, spleen, myocardium, and adrenal.[11] Ghrelin receptor has many functions including stimulate the growth of hormone secretion and appetite regulation.[9] In pancreas β-cell, stimulation of ghrelin receptor by ghrelin result decreases insulin secretion through attenuation of Ca^{2+} signaling. Moreover, low plasma ghrelin level is associated with elevation of fasting insulin level, type 2 diabetes, and insulin resistance.[12,13]

In human, single nucleotide polymorphisms (SNPs) of GHSR gene are associated with multiple phenotypes related to obesity and metabolic syndrome. Marger *et al.*[14] reported that SNP rs9819506 in GHSR gene is associated with body weight, whereas SNP rs490683 and rs509035 are associated with glucose and insulin metabolism. Baessler *et al.*[15] have described a susceptible and non-susceptible haplotype for obesity of five SNPs (rs509035, rs572169, rs519384, rs512692, and rs863441) in GHSR gene. Furthermore, Li *et al.*[16] showed an association between SNP rs2922126 or rs509030 in GHSR gene and risk of metabolic syndrome.

Many evidences of the association between the polymorphism in GHSR gene and obesity and metabolic syndrome were demonstrated in Caucasian population. However, not many evidences were obtained from Mongoloid population. The objective of this study was to investigate whether SNP rs2922126 in GHSR1a gene is associated with insulin resistance in obese female adolescents in Yogyakarta Special Region.

MATERIALS AND METHODS

Subjects

Subjects were selected based on previous study conducted in 2007. Among 137 obese female adolescents observed in the previous study, only 79 obese female met the inclusion criteria and agreed to participate in this study. The inclusion criteria was student of Junior School in Yogyakarta Special Region, obese with Body Mass Index (BMI) value according to age $\geq 95^{th}$ percentile, already had menarche, and agreed to participate in the study by signing the informed consent. The study has been approved by the Health and Medical Research Ethical Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Secondary data including name of subjects, age, body height, body weight, BMI, fasting glucose level, fasting insulin level, waist circumference and HOMA-IR index were obtained from previous study conducted by Julia *et al*.[7]

Genotype Analysis

DNA was extracted from peripheral blood using salting out method. Methods used in the genotype analysis of SNP rs2922126 were Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP).[16] TABLE 1 shows primers, PCR conditions, restriction enzymes, and digestion conditions used in the genotype analysis.

TABLE 1. Primers, PCR conditions, restriction enzymes, and digestion conditions used in genotype analysis of SNP rs2922126 GHSR1a gene

Primers	PCR conditions	Restriction enzymes	Digestion conditions
F: 5'AATCCCAGTCCTTT GTATCACTCGGT3' R: 5'AGTTATGCCTCAA AAAATGTTTCCCG3'	Forty cycles reactions of denaturation at 92 ^{0}C for 15 sec, followed by annealing for 1 min at 60 ^{0}C, and extension for 1 min at 72^{0}C.	BsrBI	Overnight at 37 ^{o}C

Polymerase Chain Reaction mixture for SNP rs2922126 consisted of 1 μL of DNA template (100 ng/μL), 10 μl kappa taq ready mix, 0.8 μL forward primer, 0.8 μL reverse primer, 7.4 μL H_2O. The total volume of PCR mixture was 20 μL. DNA amplification was performed under the conditions showed in TABLE 1. PCR products were migrated on 2% agarose gel electrophoresis and were visualized under ultraviolet light using digital imaging.

Restriction Fragment Length Polymorphism mixture for SNP rs2922126 consisted of 5 μL PCR products, 0.2 μL restriction enzyme, 2 μL buffer. The total volume of the mixture for RFLP was 20 μL. The mixture of RFLP was incubated overnight at 37^{o}C. The digestion products were separated with electrophoresis used 3.5% agarose gel and then visualized under ultraviolet light using digital imaging.

Statistical analysis

Chi-square test was used to calculate odds ratio on genotype and allele of SNP rs2922126 GHSR1a gene in insulin resistance and insulin sensitive groups. One way ANOVA test or Kruskall-Wallis test or Mann-Whitney U test was used to analyze the difference of clinical and laboratory parameters on genotype or allele of SNP rs2922126 GHSR1a gene. The $p<0.05$ was considered to be statistically significant for this analysis.

RESULTS

PCR product for SNP rs2922126 was 222 bp. Genotyping of SNP rs2922126 with RFLP showed three genotypes, which were A/A, A/T, and T/T genotypes. One band, 26 bp, could not be observed. The result for the genotyping of this SNP is shown in FIGURE 1.

Genotype and allele frequencies of SNP rs2922126 GHSR1a gene in insulin resistance group and non insulin resistance group are shown in TABLE 2. The A/T genotype was the dominant genotype in SNP rs2922126 (37%). A/A genotype individuals had higher risk to develop insulin resistance, compared to A/T and T/T genotypes individuals (OR: 2.03; 95%CI: 0.54-7.57), but this result was not statistically significant ($p>0.05$). A allele carriers increased the risk of insulin resistance compared with T allele carriers (OR: 1.36; 95%CI: 0.72-2.58), even though it was not statistically significant ($p>0.05$).

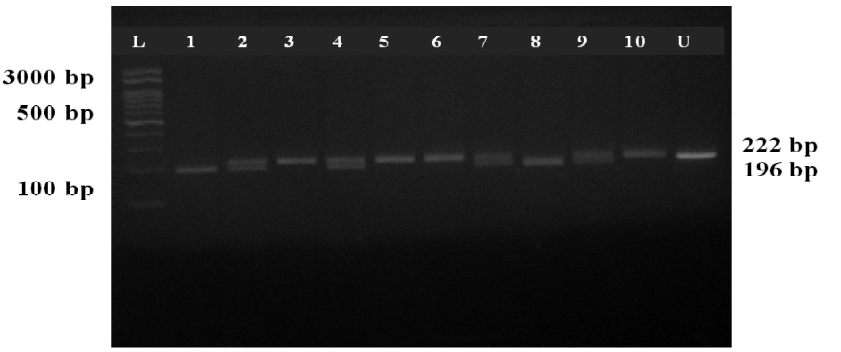

FIGURE 1. Electrophoresis of digestive product of SNP rs2922126 GHSR1a gene in gel agarose 3,5%. L: DNA ladder 100 bp; Columns 1 and 8: represented A/A homozygous genotype due to the complete cutting of the PCR product (222 bp) into two fragments (196 dan 26 bp); Columns 2, 4, 7, and 9: represented A/T heterozygous genotype (222, 196, dan 26 bp); 3, 5, 6, dan 10: represented T/T homozygous genotype due to the uncut PCR product (222 bp); U: uncut (PCR product of SNP rs2922126 GHS-R1a gene).

TABLE 2. Genotype and allele frequencies of SNP rs2922126 GHSR1a gene in insulin resistance and non insulin resistance groups

	Frequencies (%)	IR (%)	NIR (%)	OR (95%CI)	p
Genotype	78 (100)	44 (56.4)	34 (43.6)		0.40[a]
A/A	17 (21.8)	12 (70.6)	5 (29.4)	2.03 (0.54-7.57)	0.28[b]
A/T	37 (47.4)	19 (51.4)	18 (48.6)	0.89 (0.32-2.50)	0.83[b]
T/T	24 (30.8)	13 (54.2)	11 (45.8)	1.00 (Reference)	
Allele	156 (100)	88 (56.4)	68 (43.6)		0.34[a]
A	71 (45.5)	43 (60.6)	28 (39.4)	1.36 (0.72-2.58)	0.34[b]
T	85 (54.5)	45 (52.9)	40 (47.1)	1.00 (Reference)	

IR = Insulin Resistance; NIR = Non Insulin Resistance; OR = Odds Ratio; CI = Confidence Interval; [a] = The p value for Hardy-Weinberg balance, which was calculated using Chi-square test (p>0.05); [b] = p value was calculated with Chi-square test (p<0.05).

This study showed that A/A genotype individuals (TABLE 3) and A allele carriers (TABLE 4) in SNP rs2922126 had higher BMI, fasting glucose concentration, fasting insulin concentration, HOMA-IR, and waist circumference compared to other genotypes and allele, although the difference was not statistically significant (p>0.05).

TABLE 3. BMI, fasting glucose concentration, fasting insulin concentration, HOMA-IR, and waist circumference (mean±SD) based on genotype of SNP rs2922126 in GHS-R1a gene

Clinical and laboratory parameters	A/A (n = 17)	A/T (n = 37)	T/T (n = 24)	p
BMI (kg/m^2)	30.70±3.26	29.18±2.26	29.71±2.44	0.15[a]
Fasting glucose level (mg/dL)	95.70±31.01	86.16±13.85	86.25±8.02	0.50[b]
Fasting insulin level (μIU/mL)	21.24±12.78	16.66±12.01	16.86±7.40	0.18[b]
HOMA-IR	5.03±3.36	3.73±3.92	3.54±1.45	0.96[b]
Waist circumference (cm)	90.82⊥8.58	88.80⊥5.87	88.52⊥5.92	0.81[b]

[a] =p value was calculated with one way ANOVA test (p<0.05); [b]= p value was calculated with Kruskal-Wallis test (p<0.05)

TABLE 4. BMI, fasting glucose concentration, fasting insulin concentration, and HOMA-IR (mean±SD) based on allele of SNP rs2922126 in GHS-R1a gene

Clinical and laboratory parameters	A (n – 71)	T (T – 85)	p
BMI (kg/m^2)	29.91±2.84	29.48±2.35	0.55
Fasting glucose level (mg/dL)	90.73±23.69	86.21±10.84	0.68
Fasting insulin level (μIU/mL)	18.86±12.43	16.77±9.60	0.26
HOMA-IR	4.35±3.67	3.62±2.79	0.19

p value was calculated with Mann-Whitney U test (p<0.05)

DISCUSSION

This study showed that individual with A/A genotype of SNP rs2922126 in GHSR1a gene had two times greater risk of insulin resistance. These results were supported by the fact that individual with A/A genotype and A allele carrier tended to have higher BMI, fasting glucose level, fasting insulin level, HOMA-IR index, and waist circumference compared to other individuals. However the difference was not significant.

The results of this study were supported by the previous study conducted in Chinese population. Polymorphism of GHSR is associated with female metabolic syndrome in Chinese population. Individual Chinese woman with A/A genotype in SNP rs2922126 has a higher risk of metabolic syndrome associated with the increase of waist circumference and higher fasting blood glucose.[16] Moreover, this study also showed that frequencies distribution of all the genotype in SNP rs2992126 on a female adolescent in Yogyakarta was almost equal to the frequencies distribution in the Chinese woman (A/A: 21.8% vs 25.1%; A/T: 47.4% vs 45.5%; T/T: 30.8% vs 29.4%).

A/A genotype variant in SNP rs2922126 located in promoter region of GHSR1a gene might contribute to metabolic syndrome in Chinese women. This variant can alter transcriptional activity of this gene that plays an important role in the regulation of food intake and energy homeostasis. However, further researches are needed on different geographical

and racial background.[16] Study concerning the effect of SNP rs2922126 in promoter region on the expression of GHSR1a gene has not been conducted, yet, although another GHSR genetic variant, namely G/G genotype in SNP rs2922126 located in promoter region of GHSR1a gene has been conducted. This variant can increase expression of GHSR gene and lead to increase receptor signaling and thereby increasing appetite.[14] Another study reported that mutation of 151C/T in GHSR promoter may enhance transcriptional activity of GHSR gene. It can increase GHSR gene expression and GHSR signaling, causing increased appetite and decreased energy utilization.[17]

In this study, high individual variation as shown with a high standard deviation in fasting blood glucose level, fasting blood insluin level and HOMA-IR were observed. The high individual variation in these variables may cause no significant association between SNPs with insulin resistance status. Further studies involving larger number of subjects are needed to confirm this association.

CONCLUSION

In conclusion, there is no association between SNP rs2922126 within GHSR1a gene with insulin resistance in obese female adolescents in Yogyakarta Special Region.

ACKNOWLEDGEMENTS

The authors would like to thank all subjects who participated in this study.

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