Age Stratification in Genetic Variation of Lipoprotein Lipase in Metabolic Syndrome Javanese Ethnic of Indonesia

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Introduction

Metabolic syndrome (Met-S) is a common metabolic disorder and increases the risk for type 2 diabetes mellitus, cardiovascular disease and non-cardiac vascular diseases including stroke, carotid artery disease, peripheral artery disease, chronic kidney disease, atherosclerotic renal artery stenosis, and abdominal aortic aneurysms [1]. The National Cholesterol Education Program Adult Treatment Panel – III (NCEP ATP-III) guidelines and many others have defined Met-S as a combination of three or more of the following components: increased waist circumference (WC), elevated triglycerides (TG), reduced high-density lipoprotein – cholesterol (HDL-C), elevated blood pressure (BP) and elevated fasting plasma glucose (FPG) [2].

Heritability can cause Met-S and with estimates ranging from 10 to 30% [3]. Lipoprotein Lipase (LPL) is involved in the metabolism of lipids. Mature LPL is a 448 amino acid glycoprotein with chromosomal location 8p22, consisting of 10 exons and 9 introns [4]. Several mutations and variations have been described in the LPL gene in humans, most of which lead to decreased LPL-activity. Variation in LPL altering enzyme activity may confer susceptibility for or have a protective effect against triglyceride level increase, Met-S and coronary arterial diseases (CAD) [7].
Many reports described variations and missense mutations in LPL, which are clustered in several restriction fragment length polymorphisms (RFLPs). An LPL+495 T > G and PvuII C > T are common variants of the LPL gene and may be associated with subtle alterations in plasma lipids [8]. Variation of LPL+495 T > G which results from a Thymine (T) to Guanine (G) substitution and PvuII C > T which results from a Cytosine (C) to Thymine (T) substitution located on intron 6, are located 1.57 kb from the Splicing Acceptor (SA) site. This polymorphism is the product of a change of cytosine for thymine [9].

This study aimed to identify the role of LPL +495 T > G and LPL PvuII C > T gene variations in subjects with Met-S in Javanese ethnic based on age stratification.

**Methods**

**Design and participants**

This study used a case-control design. Met-S and control participants were Javanese ethnicities matched by gender and age, consisting of 80 cases and 80 control subjects (34 males and 46 females, respectively), who were recruited from a local regency in Yogyakarta, Indonesia. Inclusion criteria for the Met-S group were: subjects 20-65 years, diagnosed Met-S according to criteria of NCEP ATP III consisting of WC ≥ 90 cm for men and ≥ 85 cm for women, BP ≥ 130 / 85 mmHg or in the treatment, TG level ≥ 150 mg/dL or in the treatment, HDL-C level < 40 mg/dL for men and < 50 mg/dL for women or under treatment and FPG ≥ 100 mg/dL or treatment [1]. Inclusion criteria for control subject were: 20-65 years and not diagnosed with Met-S according to criteria of NCEP ATP III. The study was approved by the Medical and Health Ethics Committee (MHERC) of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada-Dr. Sardjito General Hospital with registration number KE/FK/0761/EC/2018. All participants in this study signed an informed consent form.

**Medical history and anthropometry measurements**

Demographic data for all participant included age, ethnicity, history of disease, cigarette and alcohol consumption. Anthropometry measurements included weight (kg), height (cm) and waist circumference (cm). Weight was measured using a digital scale by Karada Scan (Karada Scan HBF 375, by Omron) in light clothing without shoes and socks. Height was measured using stadiometers in standing position barefoot with feet together and arm by the side. WC was measured with tape on the midline between the inferior cost of the rib and the superior of the iliac crest. BMI was calculated from height and weight (kg/m2).

Blood pressure (mmHg) was measured twice by mercury sphygmomanometer in the brachial artery above the intercostal fossa after resting for at least 3 minutes. The mean of the two measurements was used for systolic and diastolic blood pressures [11].

**Biochemical measurements**

Biochemical analyses using peripheral blood was collected in EDTA blood tubes after 8 hours of fasting. FPG concentrations, total cholesterol (TC), TG, and HDL-C were measured enzymatically with an automated analyser (Cobas c111\textsuperscript{R} analyser with the protocol of Glucose HK, HDL cholesterol Gen4, Triglycerides, Cholesterol Gen2 from Roche diagnostic\textsuperscript{R} Germany) with standard protocol.

**DNA Extraction**

DNA was extracted from buffy coat using the FavorPrep\textsuperscript{TM} blood genomic DNA extraction mini Kit (Favorgen) and stored at -20°C. The concentration of DNA extraction used nanodrop to check the purity of DNA with the ratio of absorbance at 260 nm and 280 nm. A ratio of ~ 1.8 is generally accepted as “pure” for DNA.

**Genotyping and LPL Gene Polymorphism Analysis Digestion**

Genotyping of the LPL +495 T > G and PvuII C > T were performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism assays (PCR-RFLP). PCR amplification was performed in a Thermal Cycler 1000 instrument (Bio-Rad) using 30 mL of total volume. Amplification follow an initial denaturation at 95°C for 7 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 54°C (HindIII) and 58°C (PvuII), respectively for 1 minute, and extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. The primer set is shown in Table 1.

**Table 1: Primer set**

| Primers | Enzyme and palindromic sequence | Restriction (bp) | Allele |
|---------|--------------------------------|-----------------|--------|
| +495T>G | HindIII 5'-AAGCTT-3' 5'-TCCGA'A-3' | 138 and 214 | T |
| F: 5'-GATGGCTAATCTGGATAACCAAG-3' | 352 | G |
| R: 5'-CAGCTAGACATTGCTAGTGT-3' | |
| PvuII C>T | F: 5'-GCTTAATTCTCAATTCAATGTC | 188 | C |
| R: 5'-TTTAGAAGCTTGGTCCAGGT-3' | 150 and 38 | T |

In LPL +495 T > G, the PCR product was digested with HindIII enzyme using 5 U of the enzyme at 37°C for 4 hours. In LPL PvuII C > T, using PvuII
enzyme 5 U at 38°C for 1 hour. Hind III and Pvu II enzyme were purchased from New England BioLabs (NEB).

**Statistical analysis**

Data analysis was performed for normality using Kolmogorov-Smirnov tests. Differences in baseline characteristics between Met-S and control used independent T-test if data had a normal distribution and were expressed as mean ± standard deviation and Mann Whitney test if not normally distributed with median (min-max) by 95% confidence interval (CI). The Hardy-Weinberg equilibrium was tested by a goodness-of-fit with the χ² test to compare the observed genotype frequencies with the expected ones among the Met-S subjects. The frequencies of the alleles and genotypes among different subgroups were compared by the chi-square test. Then, if bivariate analysis results were significant, they were estimated by adjusted Odds Ratio (OR) and their 95% CI from logistic regression analyses with the adjustment for BMI and hypercholesterolemia.

### Results

Baseline characteristics between Met-S and control subjects are significantly different in Body Mass Index (BMI), Waist Circumference (WC), Blood Pressure (BP) and metabolic profiles (Table 2). The total numbers of subjects in this study were 160, consisting of 80 patients diagnosed with Met-S (case group) and 80 subjects as controls. The selection of case and control subjects in this study were matched by age and sex. Table 1 shows the WC, BMI, systolic and diastolic blood pressure, TG and TC were significantly higher in the Met-S group (p < 0.001) as compared to controls.

**Table 2: Demographic Characteristics of the Subjects in Met-S and control**

| Baseline characteristic | Met S (n = 80) | Control (n = 80) | P |
|-------------------------|---------------|-----------------|---|
| Aged                    | 44.73 (22.06-63.93) | 44.80 (20.46-62.49) | 0.883** |
| Gender                  |               |                 |   |
| Man                     | 34 (50)       | 34 (50)         |   |
| Woman                   | 46 (50)       | 46 (50)         |   |
| BMI (kg/m²)             | 29.97 ± 4.74  | 24.29 ± 3.62    | <0.001* |
| WC (cm)                 | 96.03 ± 10.39 | 81.71 ± 9.35    | <0.001* |
| SBP (mmHg)              |               |                 |   |
| Man                     | 130.05 (100.00) | 110.00 (90.00)  | <0.001** |
| Woman                   | 130.05 (100.00) | 110.00 (90.00)  | <0.001** |
| DBP (mmHg)              | 85.00 (60.00-145.00) | 75.00 (60.00-95.00) | <0.001** |
| FPG (mg/dL)             | 69.20 (44.05-227.60) | 69.45 (43.20-171.00) | 0.167** |
| Triglyceride (mg/dL)    | 183.72 ± 94.74 | 102.92 ± 41.09  | <0.001* |
| TC (mg/dL)              | 182.80 (88.10) | 155.15 (95.30)  | <0.001** |
| HDL-C (mg/dL)           | 35.00 (24.40-51.10) | 41.90 (22.70-76.70) | <0.001** |

*Independent sample t-test, data presented in the mean ± SD; **Nonparametric using Mann-Whitney test, data is presented in median (min-max); ***χ²-square test; p value is significant < 0.05; BMI = Body Mass Index; DBP = Diastolic blood pressure; FPG = Fasting Plasma Glucose; HDL-C = High-Density Lipoprotein Cholesterol; SBP = Systolic blood pressure; TC = Total Cholesterol; WC = Waist Circumference.

The LPL +495 T > G genotypes were determined by Polymerase Chain Reaction (PCR) and digestion using HindIII restriction enzyme (Figure 1).

![Figure 1: Results of PCR-RFLP gene variation of LPL +495T > G; M as a marker with 100 bp; lane 1, showed PCR product 188 bp; lane 2-7 showed GG genotype with 1 band at 352 bp; Lane 3-4 showed TG genotypes and consisting of 3 bands each 352 bp, 214 bp and 138 bp; Lane 5-6 showed TT genotypes with 138 bp and 214 bp.](image)

The LPL Pvu II C > T genotypes were determined by PCR and digestion using PvuII restriction enzyme (Figure 2).

![Figure 2: PCR-RFLP results of LPL PvuII C > T gene variation; M as a marker with 100 bp; lane 1, showed pPCR product 188 bp; lane 2-7 showed CC genotype with 1 band at 188 bp; Lane 4-5 showed TC genotypes and consisting of 3 bands each 188 bp, 150 bp and 38 bp; Lane 3-6 showed TT genotypes with 2 bands 150 bp and 38 bp.](image)

The population was in Hardy-Weinberg Equilibrium (p > 0.05), for LPL +495T > G and Pvu II C > T gene variation (Table 3).

**Table 3: Hardy-Weinberg Equilibrium in LPL +495 T > G and Pvu II C > T**

| Gene variation | Observed value | Expected value | χ² (DF) | P value |
|----------------|---------------|----------------|---------|---------|
| LPL +495T > G |               |                |         |         |
| TT            | 98            | 101            | 1.84    | 0.175   |
| TG            | 58            | 52             |         |         |
| GG            | 4             | 7              |         |         |
| PvuII C > T   |               |                |         |         |
| TT            | 77            | 76             | 0.06    | 0.816   |
| TC            | 67            | 68             |         |         |
| CC            | 16            | 15             |         |         |

DF = 1; χ² = Chi squared test.
Table 4: Genotype and Allele Distribution of LPL + 495T> G Genes in Met-S and Control Subjects

| Genotype / Additive Model | Met-S N (%) | Control N (%) | P | OR (CI 95%) |
|--------------------------|-------------|---------------|---|-------------|
| TT                       | 26 (32.5)   | 32 (40.0)     | Ref |             |
| TG                       | 51 (63.8)   | 47 (58.8)     | 0.384* | 0.749 (0.390-1.437) |
| TT / TG                 | 3 (3.8)     | 1 (1.2)       | 3.355** | 2.765 (0.278-27.51) |

Table 5: Age Stratification in the distribution of Genotype and Allele in LPL + 495 T > G Gene Variations in Age-related Met-S

| LPL +495 T > G | Met-S n (%) | Control n (%) | P | OR (CI 95%) |
|----------------|-------------|---------------|---|-------------|
| Met-S         | 63 (78.5)   | 37 (46.1)     | Ref |             |
| Control       | 18 (23.5)   | 50 (62.5)     |      |             |

Table 6: Genotype and Allele Distribution of LPL PvuII C>T Genes in Met-S and Control Subjects

| LPL PvuII C>T | Met-S n (%) | Control n (%) | P | OR (CI 95%) |
|---------------|-------------|---------------|---|-------------|
| TT            | 38 (47.5)   | 39 (48.8)     | Ref |             |
| CC            | 10 (12.5)   | 8 (10.0)      | 0.14* | 1.026 (0.350-3.013) |

In the ≤ 45 years age group, the distribution of TT genotypes was a greater compared to TG and GG genotypes frequency in the Met-S group although not statistically significantly different. Comparison of dominant and recessive models also found no statistically significant differences between the Met-S and control group. In group > 45 years, no GG genotypes were found in either Met-S or control subjects. Also, the distribution of genotypes and alleles of LPL + 495T > G gene variation with Met-S in subjects aged > 45 years there had no statistically significant differences.

Genotype and allele frequencies for LPL PvuII C > T were not significantly different between Met-S and controls (Table 6). The most frequent genotype was TT in both study populations, with 38% in the Met-S group and 39% in controls. In LPL PvuII C > T, the genotype TC showed the most frequent in the Met-S group compared to controls but it was not significantly different.

Age stratification in LPL +495 T > G, showed no significant differences between genotype additive, recessive and dominant models in the Met-S group and controls (Table 5).

In the age group > 45 years, the distribution of the TT genotypes and T on LPL PvuII > T gene variations age group ≤ 45 years had a greater frequency in the Met-S group compared to TC and CC genotypes although no statistically significant differences were found. In the age group > 45 years, the distribution of TT genotypes in LPL PvuII C > T gene variation was higher in the Met-S group compared to TT genotypes.

In the age group > 45 years, multivariate analysis showed a value of p > 0.05 in the Hosmer-Lemeshow test.
and Lemeshow test (Table 8). Simultaneously, obesity and genetic variation could contribute to causing Met-S as much as 41.6%. Obese patients had 13.55 times increase risk of Met-S compared to the non-obese subject and the result was statistically significant. Subjects with TC + CC genotypes had 3.62 times higher risk of Met-S compared to TT genotype.

**Table 8: Multivariate logistic regression analysis of the relationship between Pvu II C > T gene variation and Met-S at age > 45 years**

| Variable          | B     | SE    | P      | Adjusted OR (95% CI) |
|-------------------|-------|-------|--------|----------------------|
| Obesity           | 2.606 | 0.590 | < 0.001| 13.55               |
| Genotype          |       |       |        | (4.27-43.03)         |
| TT                | Ref   |       |        |                      |
| TC + CC           | 1.287 | 0.588 | 0.029  | 3.62                 |
|                   |       |       |        | (1.14-11.48)         |

**Discussion**

A study of the relationship gene variation in the LPL shown different result and the regulation of lipid concentration is complex [12]. Met-S is associated with genetics and can be influenced by the environment. Several genetic studies show different results, and this is likely because Met-S is not only caused by one type of gene but is also caused by the interaction of several genes and is influenced by environmental factors. Also, population genetic studies shown ethnic differences are one of the factors that influence differences in genotype frequency found in both LPL + 495T > G and PvuII > T gene variations. For example, food intake that may be very different between ethnic groups modulates the genetic influence on lipid metabolism [13]. Variations of the LPL + 495T > G and PvuII C > T, which are located respectively in introns 8 and intron 6, have host elements that regulate gene transcription and translation [14]. Although introns do not encode amino acids, they play an important role in processing mRNA precursors and the incorporation of exons as protein-encoding [12].

LPL +495 T > G gene variation, and the frequency of TT genotypes in some countries are more prevalent in most case groups compared to control groups such as Egypt (45%) [15], Saudi Arabia (50.8%) [8], Iran (58.8%) [16], India (48.5%) [17], Iran (56, 5%) [18] and in this study (63.8%). In this study, TT frequencies were more common in controls compared to cases. Lipoprotein Lipase Pvu II C > T gene variation in this research was the same as research conducted in the populations in Turkey, Saudi Arabia, Korea and Egypt [6], [8], [19], [20]. [19]. also reported that TC genotypes had a higher frequency in patients with coronary artery disease compared to the control group.

In the age group > 45 years, in the dominant model, TC + CC genotypes can increase the risk of Met-S by 2.567 (95% CI: 1.03-6.40). After multivariate analysis, it was found that TC + CC genotypes increased the risk of Met-S by 3.62 times compared to TT genotypes. A similar study was done by Pereira et al., in stroke patients in Colombia with a median age of 64 years without analysing food intake data and physical activity found that TT genotypes and T alleles were protective factors for stroke events [14]. This is different from the research conducted by Shin et al. which found no association between food intake and physical activity with variations in the LPL PvuII C > T gene in the population in Korea. In the study, TT genotypes showed an increase in triglyceride levels and a decrease in HDL and TT + TC genotypes with 1.5 times increased risk of Met-S compared to CC genotypes. Decreased LPL due to gene variation causes inhibition of lipolysis. Inhibition of lipolysis causes increases in VLDL and chylomicron. Furthermore, the degradation of triglycerides in adipose tissue results in an increase in free fatty acids in the blood. Increased triglycerides are caused by a decrease in the utilisation of lipids by tissue and then can cause Met-S [6]. The differences in this research with previous studies include the lack of a large number of samples, ethnic differences and several environmental factors such as physical activity and food intake that are not controlled for the research subjects. The number of samples used in the study will affect the strength of the statistical results so that the study of the relationship of gene variation to an illness requires a greater number of samples. Also, more control is needed on environmental factors that can affect the results.

There was no association between LPL +495 T > G gene variation with Met-S. In LPL PvuII gene variation, TC + CC is the risk genotype of Met-S in the age group > 45 years but no significantly different in the age group ≤ 45 years.

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