Endothelial Nitric Oxide Synthase 3 (eNOS3) Gene Polymorphisms and Essential Hypertension in Javanese Ethnic Group

Polimorfisme Gen Penyandi Endotel *Nitic Oxide Synthetase 3* (eNOS3) dan Hipertensi Esensial pada Etnis Jawa

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ABSTRACT

Hypertension is still a major public health problem in Indonesia and in several other countries. This disease is caused by multi factorial components involving both environmental and genetic factors. eNOS3 gene is one of the enzymes related to the high prevalence of hypertension. This gene expresses the NOS enzyme which regulates the synthesis of NO. NOS enzyme causes vasodilatation which decreases peripheral resistance and lowers blood pressure. This cross sectional study compared hypertension patients to those with normal blood pressure in the age group of 40-80 years old. The main purpose of this study is to evaluate the influence of eNOS3 gene Glu298Asp allele expression in Javanese ethnic group patients with hypertension. The samples consist of 50 respondents with hypertension and 50 respondents with normotension as control. Data of eNOS3 gene polymorphisms and NO plasma levels from the respondents were analyzed using t-test and chi-square test. Glu298Asp allele genotype variation in eNOS3 gene was detected by PCR-FRLP using primers G894TF and G894TR and the PCR products were cut using Mbol restriction enzymes. Sequencing result of each polymorphism band shows a typical nucleotide sequence compared to the nucleotide sequence of eNOS3 gene in Gen Bank. The results of this study showed no connection between Glu298Asp allele polymorphism in eNOS3 gene with hypertension in Javanese. There was also no relation between eNOS3 gene polymorphisms with high levels of respondents' NO plasma. Average NO plasma level of hypertension patients is 34,53 µmol/L, whereas average NO level of normal blood pressure is 32,5 µmol/L.

Keywords: *Allele Glu298Asp, eNOS3 gene, G894T, hypertension, Javanese ethnic, NO plasma level*

ABSTRAK

Hipertensi masih menjadi masalah kesehatan masyarakat yang utama di Indonesia dan beberapa negara lainnya. Penyakit ini disebabkan multifaktor yang melibatkan faktor lingkungan dan genetik. Gen eNOS3 adalah salah satu enzim yang berhubungan dengan tingginya prevalensi hipertensi. Gen ini menyandi enzim NOS yang mengatur sintesis NO. Enzim NOS menyebabkan vasodilatasi, menurunkan resistensi perifer dan menurunkan tekanan darah. Penelitian ini bersifat *cross sectional* membandingkan pasien hipertensi dengan pasien normal pada kelompok usia 40 – 80 tahun. Tujuan utama dari penelitian ini adalah untuk mengevaluasi pengaruh ekspresi gen eNOS3 alel Glu298Asp pada pasien etnis Jawa dengan hipertensi. Sampel terdiri dari 50 responden hipertensi dan 50 responden normotensi sebagai kontrol. Data polimorfisme gen eNOS3 dan kadar NO plasma dari responden dianalisis menggunakan uji t-test dan uji chi-square. Variasi genotipe alel Glu298Asp pada gen eNOS3 dideteksi dengan PCR-FRLP menggunakan primer G894TF dan G894TR dan produk PCRnya dipotong menggunakan enzim restriksi MboI. Hasil sekuensing dari masing-masing pita polimorfisme menunjukkan urutan nuklotida yang khas jika dibandingkan dengan urutan nukleotida gen eNOS3 di Gen Bank. Hasil penelitian ini menunjukkan tidak ada hubungan antara polimorfisme alel Glu298Asp pada gen eNOS3 dengan kejadian hipertensi pada etnik Jawa. Selain itu juga tidak ada hubungan antara polimorfisme gen eNOS3 tersebut dengan tingginya kadar NO dari plasma responden. Kadar NO plasma dari pesien hipertensi rata-rata adalah 34,53 µmol/L, sedangkan responden dengan tekanan darah normal kadar NO rata-rata sebesar 32,5 µmol/L.

Kata Kunci: Alel Glu298Asp, etnik Jawa, gen eNOS3, hipertensi, kadar NO plasma

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INTRODUCTION

Currently hypertension grips around 29,8% of the Indonesian population (1). This disease caused by multifactorial components involving both environmental and genetic factors. Clinical and experimental studies suggest that more than 90% of the hypertensive patients around the world suffer from essential hypertension (2). Many genes involved in blood pressure regulation have been screened and recognized as candidates for hypertension, such as endothelial nitric oxide synthase (eNOS) gene. This gene encodes the protein eNOS, the main source of endothelial derived relaxation factor NO under physiological conditions. An association between altered NO metabolism and hypertension has been found both in animal and clinical studies (3-6). Because endothelial NO availability is regulated at the level of synthesis, the eNOS gene is hypothesized to be a candidate for essential hypertension and draws considerable attention in many countries (7-8).

The NOS3 gene is located on chromosome 7q35-36 and consists 26 exons in spans 21 kb. Several studies have been shown three single nucleotide polymorphisms (SNPs) in the NOS3 gene be associated with hypertension, including G894T, T786C and a VNTR in intron (9-12). The G894T polymorphism, a G to T conversion at nucleotide position 894 in exon 7, results in a replacement of glutamate by aspartate at codon 298. This variant is not located in any functional consensus sequence, but computer analysis has revealed that the G894T mutation results in a conformational change in the eNOS protein from helix to tight turn [14]. The Asp variant of this polymorphism is associated with, coronary diseases (14,15), hypertension (16) and vascular responsiveness to phenylephrine (17). The Asp variant of this polymorphism is also believed to render the enzyme more susceptible to proteolytic cleavage (18).

Although previous evidence suggests that allelic polymorphism of endothelial NO-synthase (eNOS) may account for a considerable proportion of the risk of development of cardiovascular diseases of complex traits (14), the mechanism of the phenotypic expression of pathologic allelic variants of the eNOS gene in blood pressure traits still remains underexplored. In this study, we performed a carefully conducted family-based association study (90 participants) in Javanese trait in Indonesia. Our main aim was to estimate the effect of polymorphisms in eNOS on essential hypertension susceptibility in Javanese trait.

METHOD

Study Group and Inclusion Criteria

Consecutive hypertensive patients (n=49) admitted in the State Hospital of Margono Soekarjo, Purwokerto and Clinical Private Hospital in Purwokerto was selected. The criteria for inclusion were: Javanase race and absence of degenerative, systemic and chronic diseases, myocardial revascularization surgery, coronary angioplasty and previous myocardial infarction. The control group consisted of 49 normotensive individuals. Hypertensive patients were diagnosed after presenting a constant systolic arterial pressure, confirmed in three following measurements, higher than 140 mmHg and a diastolic arterial pressure higher than 90 mmHg. All of the procedures, risks and potential benefits were properly explained to all of the patients before they provided formal informed consent. This study with human subjects was approved by the regional bioethics committee at the Government of Indonesia.

Blood Platelet Isolation

10 ml of venous blood was taken from every subject in sterile conditions into a Falcon tube containing EDTA potassium salt (11,7 mM) as an anticoagulant (Sarstedt). Plasma and platelets were separated by centrifuging (1000 × g) of whole blood for 5 min. Platelets remain on the bottom than resuspension in Tyrode buffer containing: 137 mM NaCl, 12 mM NaHCO3, 2 mM KCl, 0,34 mM Na2HPO4, 1 mM MgCl2, 5,5 mM glucose, 5 mM Hepes (pH 7,3), containing 0,35% bovine serum albumin. Plasma in supernatant collected to measure nitrite concentration

DNA Extraction

Packed cells were lysed with Red Cell Lysis buffer. The solution was centrifuged at 4000rpm for 15min at 4°C to pellet out the nucleated cells i.e. WBCs. Nucleated cells were subjected to detergent (10% SDS) and protease (Proteinase K) treatment in Sodium Chloride-EDTA buffer and left at 37°C overnight on a shaker. Subsequently proteins were salted out with 5M NaCl. Proteins were pelleted out by centrifugation at 4000 rpm at room temperature for 15 min. DNA was precipitated by ethanol addition to the supernatant. DNA isolated is stored in TE buffer and stored at 4°C for further use. To measure the concentration and purity of DNA we use spectrophotometer.

Genotype Determination

A set of primers was designed to amplify a 206 bp fragment including the missense Glu298Asp variant (G894T polymorphism) [5'-CAT GAG GCT CAG CCC CAG-3'(forward) and 5'-AGT CAA TCC CTT TGG TGC TCA C-3'(reverse)]. Each PCR was carried out by using genomic DNA as the template in final reaction volume of 20 µL containing 10 mM Tris chloride pH 8,3, 50 mM KCl, 1,5 mM MgCl2, 200 µM each of the four dNTPs, 1 µM each of the primers, and 2U Taq DNA polymerase with the following cycling conditions: 94°C for 45 s, 59°C for 30 s, and 72°C for 45 s for 30 cycles in a PTC 100 (MJ research co.) DNA thermal cycler. The PCR fragment digested three hours at 37°C with the *Mbo*I restriction enzyme and then separated by electrophoresis on 5% agarosa gel and visualized by ethidium bromide.

eNOS Activity

For an assay of eNOS activity we used a ELISA based on Griess method. Nitrite levels were measured using a commercial Griess reaction kit (Cayman Chemicals, Ann Arbor, MI). The principle of fluorescence of triazolofluoresceine, which is formed after interaction of NO with 4,5-diaminofluoresceine, which is formed from 4,5-diaminofluoresceine diacetate (DAF-2A) under the action of intracellular esterase. The wave length of excitation/emission was 492/515 nm. The NOS inhibitor diphenyliodonium chloride (100 µM) inhibited the reaction and this confirms the specificity of the NOS activity assay. Enzyme activity was evaluated in units of fluorescence (UF) per min per 106 cells. In mammals there are few cell types, which express only one isoform of NO synthase. In platelets under normal conditions only endothelial NOS is expressed, which ensures that only the activity of this particular isoform

was assayed.

Statistical Analysis.

Data were expressed as means ± standard deviation (SD), median (inter quartile range), or absolute number (percentage) when appropriate. The t-test for independent samples was used to investigate differences between groups for approximately normally distributed variables. When the distribution of the variables was skewed the non-parametric *Mann-Whitney* U test was applied.

RESULTS

Table 1 summarizes the clinical and laboratorial characteristics of the 100 subjects enrolled in the present study. There were no statistically significant differences in gender, between hypertensive groups and the control group (all P N 0,05). Higher systolic and diastolic blood pressure were found in women with gestational hypertension or with preeclampsia compared with the other groups (both P b 0,05; Table 1).

Table 1. Clinical and laboratorial characteristics of respondent between Hypertension groups and Normotension (control) groups

No	Variable	Hypertension (n=50)	Normotension (n=50)
1	Gender		
	Male	16 (32%)	16 (32%)
	Female	34 (68%)	34 (68%)
2	Age (years)	60,64±9,555	56,72±9,051
3	Systolic	152,2±17	124,0±6
4	Diastolic	88,9±10	79,0±5
5	GDS	112,2 ± 46	106,1 ± 42
6	Cholesterol Total	184,6 ± 33	180,9 ± 34
7	Triglyceride	160,9 ±89	154±117
8	NO	34,53±9,69	32,5±16
9	Angiotensin	14,53±22	5,9±3,4

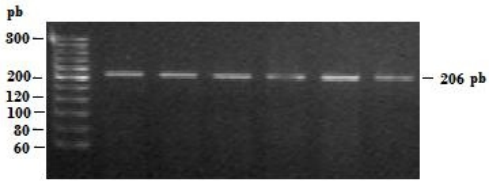

Figure 1. Electroforegram of PCR Product of fragment NOS3 gene

Note: The 206 bp band is the amplification of fragment gene NOS3

Figure 1 showed the result of amplification of fragment NOS3 gene from some genomic DNA samples. A single clear band at 206 bp is strongly indicated that fragment is belong to NOS3 gene regarding the use of specific primers. As a template, genomic DNA from blood is measured both quantity and quality with spectrophotometer. Highly quality DNA showed from the result of absorbency 260/280 close to 1.8 is used in this study (data not presented).

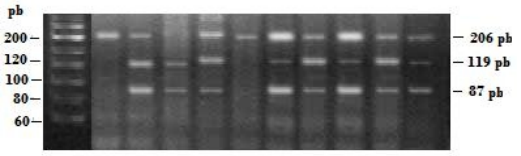

Figure 2. Electroforegram of PCR-RFLP from some samples to detect genotype variation in gene NOS3 with MboI

Note: In these figures M- marker REF-20; genotype GG cannot cutted and resulted 206 bp band. Genotype TT cutted and resulted two band 119 bp and 87 bp, while genotype GT resulted three band from uncut and cut fragment 206 bp, 119 bp and 87 bp.Due to confirming the data, after PCR-RFLP finished, some sample than sequenced to EIJKMAN Jakarta. The result of sequencing is than alignment with prefers data NOS3 nucleotide from gene bank

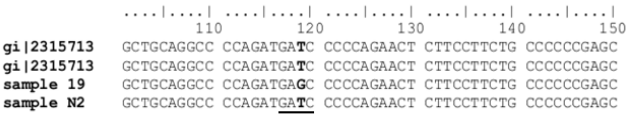

Figure 3. Sequencing product of genotypic variation on NOS3 gene

Note: Sample 19 and N2 are indicated different genotype. Sample N2 can cut with *Mbo*I but sample 19 cannot cut. GATC is a cutting site of *Mbo*I. Point mutation in this site make the enzyme cannot work. The gi2315713 is reference Sequencing of NOS3 gene from Gen bank

The distribution of genotype variation on NOS3 gene from Javanese ethnic presented in Table 2. These data indicated that genotype variation show a great effect on prevalention on hypertension (OR=2,945). Person with hypertension are dominated in genotype GT with close to 60%, but people with normal blood pressure are dominated in genotype GG with the some portion. On the other hand Genotype TT is very rare in both groups.

Table 2. Genotype and allele frequencies for NOS3 polymorphisms in both hypertension and normotension group

Polymorphism	Genotype or Allele	Hypertension N = 42		Normotension N = 45		OR (95% CI)	P
G894T	GG	16	38,1 %	29	64,4%		
	TT	1	2,4%	0	0%		
	GT	25	59,5%	16	35,6%	2,945	0,014

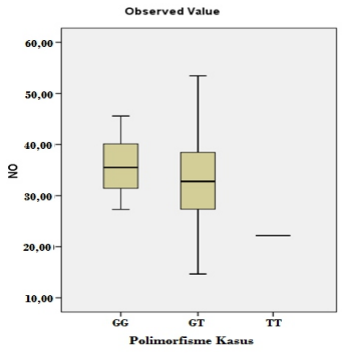

Figure 4: Intergenotypic variations in the levels of nitrite in the study subjects

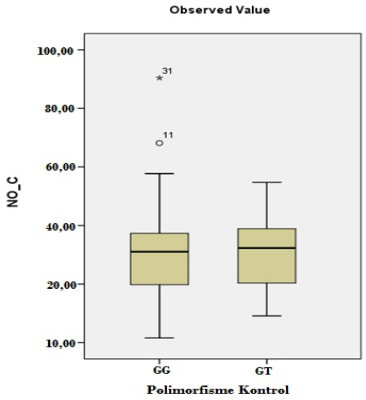

Figure 4: Intergenotypic variations in the levels of nitrite in the study subjects

Figure 4, shows that there was no significant difference in the levels of nitrite between GG and GT genotypes in both patients (p=0,66) and controls (p=0,46). However, in both the genotypes, levels of nitrite were significantly lower in patients than in controls (p<0,01 for ab and p<0,001 for bb).

DISCUSSION

The participation of females in this study was nearly threefold than that of the males. This could be due to the social forces in the society where this study was conducted. Another study done in Padang, Sumatra Barat in Minang ethnic populations, with a larger sample size showed the females subjects dominated 78,5% of distribution than male (19).

The nitrite levels were significantly the same both in patients and controls. The nitrite levels in the patients were 34,53 less higer than that in the controls. Previous studies have shown a very similar results with 43% decline in patients as compared to controls (20,21). The normal range of NO showed a wide variation in different studies (22-25). No relationship could be found between nitrite levels and gene polymorphism. This study did not find any correlation between the presence of the eNOS variant and hypertension. This may be because of the small sample size. According to the previous literature, only two studies, one on Caucasians in general (26), and another on the Ukrainian population (23), have shown a correlation between this polymorphism and essential hypertension. Other studies have found no such correlation (27,28). Although no statistically significant correlation could be found in the present study, a trend toward a higher frequency of the allele was seen among the patients with essential hypertension. However, this suggests a need to conduct a large cohort study so that the nature of any association between essential hypertension and this polymorphism can be tested. If the allele is found to be a disease-associated allele, screening of the population for individuals at risk might help save lives. If not, we can rule out an association of essential hypertension with this polymorphism. NO levels showed a significant difference between patients and controls. This suggests that an estimation of NO levels could be included as a routine lab investigation to screen people at risk and to devise appropriate individualized therapeutic strategies. However, we stress that the reference value for NO in normal Javanese trait subjects remains to be established. Estimating total NO is rather cumbersome, as it involves converting nitrate back to nitrite using the enzyme nitrate reductase. However, estimation of nitrite alone using an economic and simple method is a workable alternative.

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