

## Metalloproteinase-9 gene variants and risk for hypertension among ethnic Javanese

Fitranto Arjadi\*, Saefuddin 'Aziz\*\*, and Alfi Muntafiah\*\*\*

### ABSTRACT

#### BACKGROUND

Hypertension is associated with endothelial-dependent vasodilation disorders, due to reduced nitric oxide (NO) availability and excessive angiotensin II (ANG-II) activation. The objective of this study was to determine the association between matrix metalloproteinase 9 (MMP-9) gene polymorphism and hypertension in ethnic Javanese in the 40-80 year age group.

#### METHODS

This was a case-control study on 50 PROLANIS patients of family doctors meeting the inclusion criteria and 50 controls without hypertension. Subjects were hypertensive patients with constant systolic arterial pressure of >140 mmHg and diastolic arterial pressure of >90 mmHg, confirmed in three successive measurements. The observed parameters were degree of MMP-9 polymorphism, and NO and ANG-II levels. Matrix metalloproteinase 9 polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the SmaI restriction enzyme. MMP-9 polymorphisms were indicated by variation in band patterns. Degree of polymorphism in cases and controls were compared with NO and ANG-II levels in both groups. Data analysis was done using independent t-test.

#### RESULTS

The heterozygous (3 band) to normal (2 band) MMP-9 genotype ratio was 3:1 in hypertensives, but balanced in controls. In hypertensives, heterozygous GA and homozygous AA genotype frequencies were respectively 3.198 and 1.548 times higher than that of the GG genotype ( $p=0.008$  and  $p=0.726$ ). There was a statistically significant differences of NO and Ang-II levels between cases and controls ( $p=0.000$  and  $p=0.000$ ; respectively).

#### CONCLUSION

Matrix metalloproteinase 9 gene polymorphisms in hypertensive ethnic Javanese are associated with NO and angiotensin II levels.

**Keywords:** Hypertension, ethnic Javanese, MMP-9 gene polymorphism, adults

\*Department of Anatomy,  
Medical School,  
Medical and Health Sciences,  
General Soedirman University  
\*\*Genetics Laboratory,  
Biological Faculty,  
General Soedirman University  
\*\*\*Department of  
Biochemistry, Medical School,  
Medical and Health Sciences,  
General Soedirman University

#### Correspondence

dr. Fitranto Arjadi, MKes.  
Department of Anatomy,  
Medical School,  
Medical and Health Sciences,  
General Soedirman University  
Jl. Dr Gumbreg No 1 Mersi  
Purwokerto 53122  
Phone: +62281 622022  
Email: f.arjadi@yahoo.com

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## **Variasi genetik gen penyandi metalloproteinase-9 dan risiko hipertensi pada etnis Jawa**

### **ABSTRAK**

#### **LATAR BELAKANG.**

Hipertensi berhubungan dengan gangguan vasodilatasi yang tergantung endotel, akibat penurunan kesediaan nitrit oksida (NO) dan terjadinya aktivasi angiotensin II (Ang II), yang berlebihan. Tujuan penelitian adalah untuk menentukan adanya hubungan antara polimorfisme gena matrix metalloproteinase 9 (MMP-9) dan hipertensi pada pasien etnik Jawa usia 40 hingga 80 tahun.

#### **METODE**

Digunakan rancangan kasus control yang mengikut sertakan 50 kasus hipertensi PROLANIS dokter keluarga yang memenuhi kriteria inklusi. Kontrol merupakan pasien PROLANIS sebanyak 50 orang dengan usia 40 hingga 80 tahun yang tidak menderita hipertensi. Kasus adalah pasien hipertensi yang dicirikan dengan tekanan sistolik dan diastolik melebihi 140/90 mmHg, yang dikonfirmasi dengan tiga kali pengukuran. Parameter yang diamati adalah NO dan Ang II. Polimorfisme gena MMP-9 ditentukan dengan metode polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) menggunakan enzim restriksi SmaI. Tingkat polimorfisme antara kasus dan kontrol kemudian dibandingkan dengan kadar NO dan Ang II. Data dianalisis menggunakan uji t independen.

#### **HASIL**

Kasus hipertensi memiliki rasio heterozigot (3 pita) MMP-9 3 kali lebih besar dibandingkan genotipe normal (2 pita). Pada polimorfisme gena MMP-9, penderita hipertensi kemungkinan memiliki genotipe heterozigot GA, 3,198 kali lebih besar daripada genotip GG ( $p=0,008$ ) dan memiliki genotip AA, 1,548 kali lebih besar daripada memiliki genotip GG walaupun secara statistik tidak bermakna ( $p=0,726$ ). Terdapat perbedaan kadar NO dan Ang II yang bermakna antara kasus dan kontrol (masing-masing dengan  $p=0,000$  dan  $p=0,000$ ).

#### **KESIMPULAN**

Terdapat polimorfisme gena MMP-9 pada pasien etnik Jawa dengan hipertensi. Polimorfisme gena MMP9 berhubungan secara bermakna dengan kadar NO dan Ang II.

**Kata kunci :** Etnis Jawa, hipertensi, polimorfisme gena MMP-9, dewasa

## **INTRODUCTION**

The results of Basic Health Research (RISKESDAS) of the Ministry of Health of Indonesia in 2007 showed that hypertension has the highest mortality rate (31.9%) and that the ethnic group with the highest numbers are the Javanese.<sup>(1)</sup> In Central Java, hypertension patients rank fourth among Indonesians (37%).<sup>(2)</sup> In Banyumas, the number of hypertensive patients always higher in comparison with other diseases.<sup>(3)</sup> Many genes are involved in

hypertension, such as the matrix metalloproteinase (MMP) gene that has a higher risk in connection with peripheral resistance. Peripheral resistance leads to changes in cardiovascular structures, including myocardial hypertrophy and fibrosis resulting from changes in extracellular collagen. Matrix metalloproteinase is responsible for the balance between tissue matrix and proteolysis of extracellular matrix components such as elastin, proteoglycan and collagen, that have an important role in blood vessel remodeling.<sup>(4)</sup> A

high level of metalloproteinase-9 (MMP-9) affects the thickness of the tunica media of blood vessels and decreases the possibility of arterial stiffness.<sup>(5)</sup> A meta-analysis by Niu et al.<sup>(6)</sup> showed the connection of MMP-9 gene polymorphism with coronary heart disease.

Matrix metalloproteinase 9 is responsible for a balanced tissue matrix and contributes to vascular stiffness. Matrix metalloproteinase 9 gene polymorphisms which lead to an increase in MMP-9 can cause differences in individual susceptibility to hypertension.<sup>(7)</sup> A decrease in nitric oxide (NO) production in endothelial cells activates the extracellular matrix of the renal tubuli, namely the metalloproteinase-2 (MMP-2) matrix and MMP-9 matrix, its induction of microvascular dilation in renal glomeruli and renal tubuli in order to increase filtration and decrease blood pressure. The interstitial connective of blood vessel and capillary endothelial basement membrane contains ultrastructural elastin and collagen, but the imbalance between MMP and its inhibitor, tissue inhibitor of metalloproteinase (TIMP), will lead to increased expression of MMP-2 and MMP-9. The next process of MMP-2 and MMP-9 is degrading elastin and collagen and replacing it with more rigid oxidized collagen. This process is exacerbated in polymorphisms of the MMP-9 gene at a distance of 277 nucleotides, where guanine is substituted by adenine, so causing the MMP-9 activation to occur continuously.

Polymorphisms of the MMP-9 gene, located in the 20q12.2-13.1 chromosome, are found at promoter, coding, and untranslated regions.<sup>(8)</sup> On the other hand, angiotensin II (Ang II) and NO have an important role in the blood pressure feedback regulation mechanism. Ang II controls the expression of endothelial cell nitric oxide synthase (eNOS), which produces NO. NO synthesis results in smooth muscle cell vasoconstriction, while NO reduces the expression of Ang II receptors in endothelium causing vasodilation of blood vessels.<sup>(9)</sup>

The study of MMP-9 gene polymorphisms in ethnic Caucasians showed that the base change

C to T in the MMP-9 gene promoter at -1562 increases the risk of heart disease due to an increase in systolic and diastolic blood pressures.<sup>(10)</sup> Another study on ethnic Indians showed a correlation between MMP-9 gene polymorphism and hypertension and also the occurrence of left ventricular damage in the heart.<sup>(11)</sup> In the Brazilian population, a polymorphism of the MMP-9 gene is associated with high cholesterol levels that indicate a relation with abnormalities of fat metabolism and heart disease.<sup>(12)</sup> However, the presence of polymorphisms of MMP-9 genes in ethnic Han Chinese was not associated with the incidence of stroke or intracerebral hemorrhage.<sup>(13)</sup>

The differences in frequency of polymorphisms of MMP-9 genes with the incidence of hypertension in several human ethnic groups are interesting for further study. In Indonesia there are many ethnic groups and one of the largest are the Javanese. Besides, basic molecular analysis of polymorphisms of MMP-9 genes in hypertension patients of Javanese ethnicity has not been carried out. Therefore it is important to determine the effects of MMP-9 gene polymorphism in hypertension patients of Javanese ethnicity. The objective of this study was to determine the association between MMP-9 gene polymorphism and hypertension in ethnic Javanese in the age group of 40-80 years.

## METHODS

### Research design

An observational study with case-control design was conducted from July to December 2012. The research was carried out in the Research Laboratory of General Soedirman University in Purwokerto, Central Java.

### Research subjects

The case group consisted of 50 patients of family doctors associated with the PROLANIS program (Chronic Disease Management Program, *Program Pengelolaan Penyakit Kronis*) in Banyumas in collaboration with PT

ASKES (the National Health Insurance agency). The inclusion criteria for the case group were: male or female, ethnic Javanese (3 generations), aged  $\geq 40$  years, with blood pressure of  $>140/90$  and not taking antihypertensive medications. The control group consisted of 50 persons meeting the following inclusion criteria: male or female, ethnic Javanese (3 generations), aged  $\geq 40$  years, with normal cholesterol, triglyceride, blood glucose levels, and normal body mass index (BMI). All participants were diagnosed according to their medical history and clinical symptoms by clinicians under supervision of a consultant before being assigned to the case group or control group.

### Blood sample collection

A volume of 6 ml of venous blood from each participant was collected in a tube and used for DNA isolation and analysis of serum NO and Ang II levels. Blood for determination of NO and AngII was collected in an Eppendorf tube with anticoagulant and centrifuged at 4.000 RPM for 10 minutes, so that the yellowish serum forming the supernatant was separated from the erythrocytes. The determination of NO levels by the Griess' method by calculating nitrate and nitrite concentrations uses the formula:

$$[\text{Nitrate} + \text{nitrite}] [\text{i M}] = \frac{[\text{A } 540\text{-y}] [\text{200 } \mu\text{L}] \times \text{dilution}}{\text{slope} \quad \text{sample volume}}$$

### DNA extraction

Extraction of DNA used the method of Miller et al.<sup>(14)</sup> Packed cells were lysed with Red Cell Lysis buffer and then centrifuged at 4000 rpm for 15 min at 4°C to pellet out the nucleated cells (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. Proteins were subsequently salted out with 5M NaCl, then pelleted out by centrifugation at 4000 rpm at room temperature for 15 min. To the supernatant,

which was transferred to another tube, ethanol was added to precipitate the DNA. The isolated DNA was stored in TE (Tris-HCl EDTA) buffer in 4°C for further use. The concentration and purity of the isolated DNA was measured by spectrophotometry.

### Genotype determination

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) procedure for polymorphism of MMP-9 genes started with the amplification of MMP-9 genes by using the forward primer R279Q F: 5'- ATG GGT CAA AGA ACA GGA-3' and reverse primer R279Q R: 5'- GGT AGA CAG GGT GGA GG-3', producing 277-bp long products.<sup>(15)</sup> The PCR conditions include an initial denaturation of 95°C 5 minutes, 35 cycles consisting of denaturation 94°C 30 seconds, annealing 58°C 30 seconds, elongation 72 °C 45 seconds and final elongation 72°C 10 minutes. The PCR product was cut with the SmaI restriction enzyme. Digestion results were analyzed by electrophoresis using agarose gel 5% and visualized with ethidium bromide under UV light. AA electrophoresis results were not cut, GG was cut into 2 fragments of 181 bp and 96 bp, while CA was cut into 3 fragments of 277 bp, 181 bp and 96 bp.

### Statistical analysis

Data were expressed as means of absolute number (percentage), and independent-t test was used to determine the differences between individuals carrying polymorphisms of NOS3 genes and MMP-9 genes in hypertension patients and if the distribution was abnormal we continued with the Mann Whitney test.

### Ethical clearance

All procedures have been approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Gadjah Mada University (Approval letter reference number: KE/FK/734/EC).

Table 1. Association of MMP-9 gene polymorphisms between case and control groups

	Blood Pressure		OR (95% CI)	p
	Cases (n=50)	Control (n=50)		
<b>MMP-9 gene</b>				
AA	5 (10%)	6 (12%)	1.548 (0.4 - 5.988)	0.726
GA	31 (62%)	18 (36%)	3.198 (1.338 - 7.646)	0.008
GG	14 (28%)	26 (52%)	1	

## RESULTS

Table 1 shows the association between polymorphism of MMP-9 genes with hypertension of the 100 subjects who participated in the study. The genotypes GG and GA were separated with SmaI enzyme at its restriction sites and yielded 2 and 3 bands, respectively. On the other hand, there was no SmaI enzyme restriction site in the AA genotype, so AA appeared as 1 band. In subjects of the case group, the highest frequencies was for the GA genotype (62%), followed by the GG genotype (28%), while the AA genotype had the lowest frequencies (10%). In contrast, among the control subjects, the greatest number was in the GG genotype (52%), followed by the GA genotype (36%) and AA genotype (12%). In hypertensive, the ratio of heterozygous (3 band) to normal (2 band) MMP-9 genotype was 3:1. The control group had a balanced ratio of heterozygous and normal genotype. In hypertensive patients the frequencies of heterozygous GA and homozygous AA genotypes were respectively 3.198 and 1.548 times higher than the frequencies of the GG genotype (at  $p=0.008$  and  $0.726$ , although the latter was not statistically significant).

Statistical analysis showed that there was a significant of angiotensin II and NO levels between cases and controls ( $p=0.000$  and  $p=0.000$ ; respectively) (Table 2).

Figure 1 shows the electrophoresis results of PCR-RFLP products to detect genotype variation in MMP-9 genes. The M marker REF-20, AA with the band size of 277 bp is not cut by the restriction enzyme, GG is cut into two bands 181 bp and 96 bp, while GA is a combination of one uncut band and two cut bands, producing three bands of sizes 277 bp, 181 bp, and 96 bp.

After completion of the PCR-RFLP, some samples were sequenced at the Eijkman Laboratory, Jakarta. The MMP-9 PCR fragment products show that sample m1 is an uncut band (Figure 2). Sample k11 forms two bands because it is cut by the SmaI enzyme, while sequence gi7427228 is a human MMP-9 gene sequence from the Gene Bank. The nucleotide sequences at site 96 in bold print show the variation between sequences and the nucleotide CCGGG sequence (underlined) is that recognized by SmaI.

## DISCUSSION

Regarding MMP-9 gene polymorphism, hypertension patients may have higher frequencies of the heterozygous genotype GA (62% or 3.198 times) than homozygous genotypes (GG genotype and AA genotype). On the other hand, the group without hypertension (control) have smaller genotype frequencies of heterozygous genotype GA (18%) than

Table 2. Comparison of NO and angiotensin II levels between case and control groups

Parameters	Cases (n=50)	Control (n=50)	p
NO ( $\mu\text{m ol/L}$ )	29.53 $\pm$ 3.90	33.98 $\pm$ 7.91	0.000
Angiotensin II (pg/dl)	5.90 $\pm$ 3.40	14.53 $\pm$ 22.00	0.000

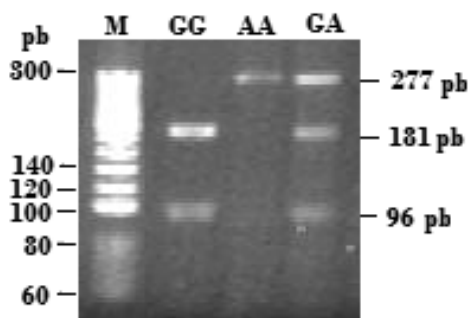


Figure 1. Electropherogram of MMP-9 gene fragment PCR products.  
Note: pb = base pair(s)

homozygous frequencies of GG (52%). This result is the same as the report on Brazilians with hypertension by Mazzoti et al.,<sup>(12)</sup> among whom the frequency of the MMP-9 heterozygous genotype reached 70.3%. However, Fan et al.<sup>(16)</sup> reported that heterozygous allele frequencies in MMP-9 polymorphisms both in the control group and case group of Chinese stroke patients showed approximately equal percentages ( $\leq 25\%$ ). Our results showed, similarly to all other studies that have been done before, that the AA genotype frequency is always the lowest, both in case and control groups. This indicates that the AA genotype is a genetic factor causing hypertension due to G->A (guanine to adenine) base changes. In the present research, the AA genotype is not visible as a clinical sign of hypertension because the MMP-9 enzyme may have vasodilator and vasoconstrictor effects. The MMP-9 enzyme is able to degrade the extracellular matrix which can lead to vasodilation in the early phase but not to vascular stiffness. The excessive action of the MMP-9 enzyme may eventually lead to continuous blood vessel stiffness, causing hypertension. Therefore the AA genotype is not a risk factor in the early stages of hypertension, but continued activity can cause MMP-9 to

become a risk factor for cardiovascular disease.<sup>(17)</sup>

The polymorphism of MMP-9 genes and the lack of tissue inhibitor of metalloproteinase (TIMP) can cause the excessive MMP-9 enzyme activity that will convert elastin and collagen into more rigid forms.<sup>(18)</sup> Polymorphism of MMP-9 in the promoter and coding sequences affects the MMP-9 levels and activity, which will strongly increase and cause arterial stiffness, both in men and women,<sup>(17)</sup> therefore the MMP-9 gene affects cardiovascular mortality, especially in patients with the 279 Gln allele.<sup>(19)</sup> The differences in age and gender also influence the response of MMP-9.<sup>(17)</sup>

Matrix metalloproteinase-9 was significantly associated with NO, which means that there are significant differences in NO levels between MMP-9 gene polymorphism groups. On the other hand, Alp et al.<sup>(20)</sup> reported that hypertension in Turkish people is not related to polymorphisms occurring both in MMP-9 and NOS3. Our study also showed that MMP-9 polymorphism was not significantly associated with angiotensin II. The result of no significant differences between polymorphism MMP-9 and Ang II in hypertension cases in our study is unclear. The tendency to increased blood pressure in the elderly subjects is a normal phenomenon unrelated to hypertension.

With respect to the emergence of coronary heart disease, NO is able to regulate MMP-9 activity through guanylyl-cyclase dependent and independent pathways, chemically through protein modification by reactive nitrogen species, biologically through modulation of MMP-9/TIMP-1 balance via soluble guanylyl-cyclase-dependent pathways, and proteolytically through the regulation of MMP-1 and MMP-13 which can cleave the prodomain of MMP-9.<sup>(21)</sup>

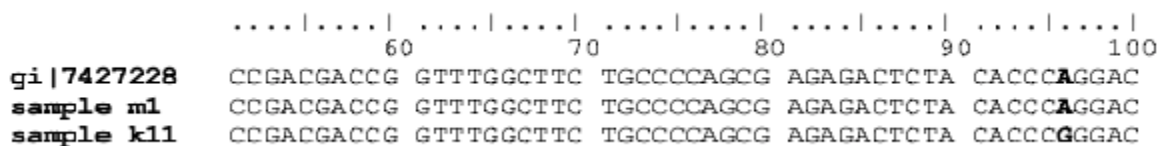


Figure 2. Sequencing products of genotypic variation in MMP-9 genes

Inhibition of nitric oxide synthase (NOS) can increase the expression of MMP-9, so decreasing NO levels will increase MMP-9 activity.<sup>(22)</sup> The increased activity of MMP-9 enzyme causes vasodilation and extremely excessive activity will lead to changes in elastic tissue in the blood vessels, causing vasoconstriction.<sup>(18)</sup> The results of previous studies on MMP-9 promoter polymorphism showed that the T allele results in a higher promoter activity compared with the C allele, because of the preferential binding of a transcription repressor protein to the C allele.<sup>(23)</sup> The T allele has also been associated with higher plasma MMP-9 levels in patients with coronary artery disease (CAD).<sup>(19)</sup>

Angiotensin II increases the expression of MMP-9 genes by inducing monocytes, through the G protein/non-G pathway, mobilization of Ca<sup>2+</sup>, mitogen-activated protein kinases (MAPK), receptor and non-receptor tyrosine kinase, Janus family kinases-signal transducers and activators of transcription-suppressors of cytokine signaling (JAK-STAT-S), small G proteins (Ras, Rho, Rac, and others), and activation of NADPH oxidase, which further increases MMP-9.<sup>(24,25)</sup> The increased levels of MMP-9 cause matrix degradation in the *tunica intima* of blood vessels that are involved in vasodilation. The condition of excessive MMP-9 enzymes alter the elastic tissue of blood vessels to become stiff and cause vasoconstriction.<sup>(26)</sup> In the present study, for some effects the explanation of MMP-9 polymorphism acting synergistically in the vasoconstriction process is not sufficient, so these effects might be influenced by other factors.

This study only examined the polymorphism in the promoter region of MMP-9 without measuring the MMP-9 enzyme levels. The age range of the subjects and the number of subjects have no significant influence on the results of our study. However, the knowledge of the relationship of MMP-9 polymorphisms, NO and AngII levels in the Javanese population in connection with essential hypertension opens

opportunities for early detection of hypertension by determination of biomarkers.

## CONCLUSION

In ethnic Javanese hypertension patients, there occurs polymorphism of MMP-9 genes, an association between polymorphism of MMP-9 genes, angiotensin and NO levels.

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